A Unique Glutamic Acid-Lysine (EK) Domain Acts as a Splicing Inhibitor*

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SRrp86 is a unique member of the SR protein superfamily of splicing factors containing one RNA recognition motif and two serine-arginine (SR)-rich domains separated by an unusual glutamic acid-lysine (EK) rich region. Previously, we showed that SRrp86 could regulate alternative splicing by both positively and negatively modulating the activity of other SR proteins as long as the entire region encompassing the RS-EK-RS domain was intact. To further investigate the function and domains of SRrp86, we generated a series of chimeric proteins by swapping the RNA recognition motif and RS domains between SRrp86 and two canonical members of the SR superfamily, ASF/SF2 and SRp75. Although domain swaps between SRrp86 and ASF/SF2 showed that the RRMs primarily determined splicing activity, swaps between SRrp86 and SRp75 demonstrated that the RS domains could also determine activity. Because SRp75 also has two RS domains but lacks the EK domain, we further investigated the role of the EK domain and found that it acts to repress splicing and splice-site selection, both in vitro and in vivo. Incubation of extracts with peptides encompassing the EK-rich region inactivated splicing and insertion of the EK region into SRp75 abolished its ability to activate splicing. Thus, the unique EK domain of SRrp86 plays a regulatory role controlling RS domain function.

The coding regions of nearly all eukaryotic genes contain intervening sequences (introns) that must be efficiently and precisely removed to allow translation of functional proteins (1). For many genes, introns are constitutively removed during the formation of mRNA, however, for a large number of genes, the removal of introns is regulated such that various combinations of exons are spliced together in a tissue-specific and/or developmentally specific fashion (2, 3). In fact, greater than 60% of known genes are subject to alternative splicing (4, 5), and it has been estimated that up to 15% of characterized genetic diseases involve mutations that cause defects in splicing (6). Many of these diseases are caused by mutations in the splice-site sequences but misregulated alternative splicing can also cause disease (7).

Before the irreversible commitment of a given pair of splice sites, introns and exons must be defined, typically through the formation of bridge complexes (8–14). A family of proteins containing regions rich in serine-arginine dipeptides (SR proteins) (15) plays an important role in bridge-complex formation and splicing by mediating protein-protein interactions across either introns or exons (8, 10–12, 14, 16). SR family members are characterized by the presence of one or two amino-terminal RNA recognition motifs (RRMs) and a carboxyl-terminal domain rich in serine and arginine dipeptides (SR). Bridge-complex interactions have been detected between SR family members (10, 11, 17–19), between SR proteins and non-SR splicing factors (20–22), and between SR proteins and putative nuclear matrix components (23). Alternatively spliced exons are often flanked by non-consensus splice sites and are activated for splicing by SR proteins binding to nearby enhancer elements facilitating the recruitment and assembly of spliceosomes (24). SR proteins are therefore crucial regulators of alternative splicing.

Because many SR family members can individually complement splicing-deficient S100 cytoplasmic extracts, it first appeared that they might be functionally redundant. Consistent with partial redundancy, blocking the expression of six different SR proteins in C. elegans using RNA interference resulted in no observable phenotype (25). However, RNA interference inhibition of ASF/SF2 in C. elegans, targeted disruption of ASF/SF2 in chicken DT40 cells, and null alleles of the Drosophila SR protein B52 all resulted in lethality (25–27). In addition, multiple studies have shown that individual SR proteins display substrate specificity, have distinct functions in alternative splicing events, and can even negatively regulate splicing (11, 17, 18, 28–44).

Domain-swap experiments have been widely used to study SR proteins and discern the function of the RRM and RS domains. Although the results of these studies are somewhat complex, the general conclusion is that RRMs are responsible for RNA binding and specificity in both alternative and constitutive splicing, and that they confer the ability to commit different pre-mRNAs to splicing (36, 45–47). In contrast, RS domains are thought to be protein-protein interaction domains that are often interchangeable.

We recently identified SRrp86, an SR-related protein of 86 kDa. SRrp86 contains one RRM at the N terminus and two RS domains at the C terminus (44). The overall primary structure is very similar to canonical SR proteins except that SRrp86 also possesses a unique glutamic acid-lysine rich domain (EK domain) between the two RS domains. SRrp86 is not required for splicing but it can affect the function of canonical SR proteins both in vitro and in vivo. It appears that SRrp86 acts to regulate the activity of other SR proteins, both positively and neg-

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1 The abbreviations used are: RRM, RNA recognition motif; RNP, ribonucleoprotein; aa, amino acid; Ni-NTA, nickel-nitrilotriacetic acid.
atively, through protein-protein interaction. Previously, we showed that SRrp86 inhibits ASF/SF2, SC35 and SRp55 whereas it activates SRp20, provided the RS-EK-RS domains are intact (44, 48). In order to understand how SRrp86 might act to both positively and negatively regulate other SR proteins, we sought to identify what domains are needed for function and to determine what role the EK region might play in overall protein function. Using in vitro and in vivo splicing assays and a series of domain-swap constructs, we found that the splicing activity of the chimeric proteins was not completely determined by their RRMs; rather, the RS domains could also direct activity. Specifically, fusion of the RS domains from SRp75 to the RRM from SRrp86 resulted in a chimeric protein capable of activating alternative splice site selection, whereas the parental SRrp86 protein was inactive. Thus, the RS domains from SRp75 can regulate splice site selection. In addition, we discovered that the novel EK domain of SRrp86 can negatively regulate both constitutive and alternative splicing, indicating a plausible mechanism of splicing regulation by SRrp86.

**EXPERIMENTAL PROCEDURES**

**Plasmid Constructs**—All chimeric constructs were cloned into the pcDNA-Amp vector (Invitrogen) for in vivo alternative splicing assays, and the pAcHLT vector (BD Biosciences) for protein expression. Briefly, individual RRM and RS domains were amplified by PCR with specific primers, digested, and ligated to generate domain swap constructs. The specific oligonucleotides and conditions are available upon request. All constructs were verified by sequencing. The amino acids (aa) included in each construct consist of: SRrp86<sup>RMM</sup>, aa 198–248 of ASF/SF2; SRrp86<sup>RMM</sup>–SRp55<sup>RS</sup>, aa 199–494 of SRrp86 replaced aa 198–248 of ASF/SF2; SRp55<sup>RMM</sup>–SRrp86<sup>RS</sup>, aa 179–494 of SRp75 replaced aa 198–494 of SRrp86; SRrp86<sup>RMM</sup>–SRp75<sup>RS</sup>, aa 198–494 of SRrp86 replaced aa 179–494 of SRp75; SRrp86<sup>ΔEK</sup>, aa 262–345 of SRrp86 were deleted; SRp75<sup>ΔEK</sup>, aa 262–

**Fig. 1.** Alignment of RRM and RS regions. The domain structure of SRrp86 and the sequence of the EK region is shown. The RRM and two RS domains from SRrp86 were aligned (63) with the indicated SR family members. Black boxes indicate amino acid identity with gray boxes representing similar amino acids. The conserved RNP-1 and RNP-2 boxes within the RRM are as indicated.

**Fig. 2.** Chimeric proteins. The RRM (RNA Recognition Motif) and RS (Arginine-Serine rich domain) domains were swapped between SRrp86, SRp75, and ASF/SF2. The relevant domains are represented in superscript. To avoid confusion, chimeric constructs derived from ASF/SF2 are simply denoted with ASF and the relevant domain. The EK domain was either deleted from SRrp86 (ΔEK) or inserted between the two RS domains of SRp75 (SRp75<sup>ΔEK</sup>).
345 from SRrp86 replaced aa 272–284 of SRp75. The EK region (aa 262–345) was also amplified and cloned into pET28a (Novagen).

Protein Expression and Purification—The pAcHILT-derived constructs were co-transfected into SF9 cells with linearized BaculoGold (Pharmingen), and recombinant virus was generated and amplified. Amplified viral stocks were then used to infect Hi5 cells. After 48–72 h, cells were diluted in fresh LB and grown to an A600 of 0.8, followed for 30 min. The cleared lysates were then incubated with Ni-NTA agarose beads and purified as previously described (44). Bound proteins were eluted with imidazole and dialyzed into splicing buffer D.

For splicing-inhibition experiments using recombinant EK peptide, a region corresponding to amino acids 262–345 was amplified by PCR and cloned into pET28a. Both pET28a-EK and, as a control, the empty vector pET28a were expressed in Escherichia coli (BL21). Overnight cultures were diluted in fresh LB and grown to an A600 = 0.8, followed by induction at a final concentration of 1 mM isopropyl-β-D-thiogalacto-side for 1 h at 37 °C. Subsequent purification by passage over Ni-NTA agarose was as described (49) followed by dialysis into splicing buffer D (20 mM Tris, pH 8, 100 mM KCl, 0.2 mM EDTA, 0.5 mM diethiothreitol, 5% glycerol).

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In Vitro Transcription and Splicing Assays—Transcription and splicing were carried out as previously described (44). The 5’-16X (44, 50), AdeML (51), and cis-parent (52) templates were linearized with BamHI and transcribed with T7 RNA polymerase (5’-16X and AdeML) or SP6 RNA polymerase (cis-parent). The 5’-16X and cis-parent substrates were spliced in HeLa nuclear extract supplemented with the indicated recombinant proteins. AdeML was spliced in either HeLa nuclear extract (Fig. 7) or a mixture of HeLa nuclear and S100 extracts (1:4) (Fig. 6). For the different recombinant protein preparations, titration experiments were first performed over a range of concentrations to ascertain the levels used. Depending on the preparation, the concentration of added protein ranged from 0.08 to 1.6 μM. After splicing, products were separated on 8% (5’-16X), 10% (cis-parent), or 15% (AdeML) denaturing gels, dried for 1 h (except for 15% gel), and analyzed on a PhosphorImager.

Cell Culture and Transfections—HeLa cells were grown in Dulbecco’s modified Eagle medium supplemented with 10% bovine calf serum, 100 units/ml penicillin and 100 μg/ml streptomycin. Transfections were performed on 60-mm 70% confluent cells using Trans IT-LT2 (Mirus) with 1 μg of SrRp20 mini-gene, 200 ng of pCDNA-SRrp20, and 200 ng of chimeric constructs. After 41 h, cells were harvested and total RNA was isolated using Tri Reagent (Molecular Research Center). Splicing patterns were analyzed by RT-PCR as described (44, 48).

Spliceosome Assembly Analysis—AdeML pre-mRNAs (5 ng) were incubated with nuclear extract/S100 mixtures (1:4) supplemented with the indicated amounts of recombinant protein under normal splicing conditions for 5 or 10 min. Samples were frozen on dry ice and thawed, and 1.25 mg of heparin was added. Splicing complexes were resolved on 4% polyacrylamide gels (acrylamide:bis; 80:1) under native conditions as previously described (53). Gels were dried for 1 h and visualized using a PhosphorImager.

RESULTS

Construction of Chimeric Proteins—Based on functional analyses, SRrp86 is an unusual member of the SR protein superfamily (44, 48). Comparison of the primary amino acid sequences from a variety of SR proteins shows that SRrp86 is structurally distinct with two RS domains separated by a unique EK-rich region (Fig. 1). To examine this further, both RS domains and the RRM from SRrp86 were aligned with other SR family members (Fig. 1). RRM regions with more highly conserved octamer and hexamer motifs referred to as RNP-1 and RNP-2, respectively. As shown, the RRM from SRrp86 contains conserved RNP-1 and RNP-2 boxes but is otherwise much less well conserved than other members of the SR protein superfamily. For the RS domains,
SRrp86 is again unusual. Both domains contain far fewer RS or SR dipeptides and the RS rich regions are much smaller than their counterparts in other SR proteins. The length of the RS domains may be important for function because SRp30c, which contains only a small RS region, is not as efficient in rescuing splicing-deficient S100 extracts as its closest family member.
The RS Domains from SRrp86 and SRp75 Are Unique—A common property of canonical SR proteins is the ability to complement splicing-deficient S100 cytoplasmic extracts (29, 57). We initially tested the ability of the parental SR proteins and chimeric constructs shown in Fig. 2 to restore splicing in S100 extracts. Although ASF/SF2 and, to a lesser extent, SRp75 were able to rescue splicing, none of the chimeric proteins could efficiently restore splicing (data not shown). This was not entirely unexpected because SRp75 cannot complement S100 extracts (44). However, because ASF/SF2 and SRp75 can alter splice site selection (30, 56, 58), and because SRp75 appears to function by modulating the activity of other SR proteins, it appears that the EK domain may act to repress constitutive splicing.

To analyze 5’ splice-site selection, a substrate derived from β-globin was used that contains duplicated, competing 5’ splice sites (5’-Δ16X). Splicing of this substrate in HeLa nuclear extract led to almost complete selection of the upstream (distal) splice site, whereas supplementation of extracts with purified ASF/SF2 (29). RS domains are known to be subject to phosphorylation, and it has been proposed that regions of alternating charge, such as would occur upon serine phosphorylation, may be necessary for SR domain function (54). SRrp86 is subject to phosphorylation in the two RS domains but the subsequent regions of alternating charge are clearly smaller than other SR proteins. The EK-rich region might be able to functionally substitute for this difference since it maintains alternating charged residues in the absence of phosphorylation. To understand the role that the RRM, RS, and EK domains contribute to overall SRrp86 function, a series of chimeric proteins were created. Domain swaps were performed between SRrp86 and SRp75 because it is the only SR family member with two RS domains, and between SRrp86 and ASF/SF2, for which considerable domain analyses have previously been reported (36, 47, 55, 56). Finally, we deleted the EK-rich region from SRrp86 (SRrp86ΔEK) and also inserted it between the two RS domains from SRp75 (SRp75+EK). The names and relevant domains included in each swap construct are detailed in Fig. 2. Each construct was subcloned into vectors for transfection studies or for purification from baculovirus-infected cells.

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exception of SRp75, none of the parental or chimeric proteins were able to significantly alter splice-site selection (data not shown). As above, the RS domains from SRrp86 could be combined with the RRM from SRp75 and activate proximal splicing but the effects were small, from 1.5- to 2-fold. Overall, it appears that the mechanisms by which these proteins affect splicing is different for 5’ versus 3’ splice-site selection.

The EK Domain Inhibits Alternative Splice-site Selection—To analyze the role of the EK region, chimeric and deletion constructs were created in which the EK domain was either inserted between the two RS domains in SRp75 (SRp75 + EK) or deleted from SRrp86 (SRrp86ΔEK). In vitro splicing assays were performed to analyze the effects of these constructs on alternative splice-site selection (Fig. 4). As shown, deletion of the EK domain from SRp75 did not affect overall splicing efficiency, but also caused a 3- to 4-fold increase in proximal 5’ splice-site selection (Fig. 4A). This was in distinct contrast to the parental SRrp86 protein, which did not significantly increase either splicing efficiency or proximal splice-site selection. Addition of the EK domain to SRp75 blocked the ability of SRp75 to activate proximal splicing.

For 3’ splice-site selection, SRrp86 was unable to alter splicing, and deletion of the EK domain had no effect (data not shown). However, insertion of the EK domain into SRp75 blocked the ability of SRp75 to activate proximal 3’ splice-site selection (Fig. 4B). Although these results again argue that the mechanisms governing 5’ versus 3’ splice-site selection are distinct, they also suggest that the EK domain may act as a repressor. The difference between SRp75RRM-SRrp86RR (which was able to regulate alternative splicing in Fig. 3) and SRp75–EK indicates that the context of EK domain is also important.

The EK Domain Inhibits in Vivo Alternative Splicing—The inhibitory effect of EK domain on in vitro alternative splicing prompted us to examine its function in vivo. The gene encoding SRp20 consists of 7 exons and is subject to autoregulatory alternative splicing with exon 4 included or excluded (Fig. 5A) (42). Exon 4 inclusion leads to the production of a truncated protein due to the presence of a premature stop codon. Excess SRp20 results in increased inclusion of exon 4, down-regulating production of full length SRp20. Previously, we showed that SRrp86 activates SRp20, leading to increased inclusion of exon 4 (48). We therefore used the same SRp20 mini-gene as a reporter to characterize the function of the EK domain during in vivo splicing. Transfection of HeLa cells with the SRp20 mini-gene resulted in greater than 99% skipping of exon 4 whereas co-transfection of an SRp20 cDNA increased the level of exon 4 inclusion (compare band ratios between the first two lanes of Fig. 5B). Co-expression of SRrp86 with SRp20 further increased exon 4 inclusion about 3- to 4-fold, whereas co-expression of SRp75 with SRp20 caused a 2- to 3-fold increase (Fig. 5, B and C). With these baselines in hand, we tested the ability of SRrp86ΔEK and SRp75 + EK to activate exon 4 inclusion when co-transfected with SRp20 cDNA. Fig. 5B shows a representative gel for the parental and chimeric proteins, and the fold increase from multiple experiments is shown in Fig. 5C. From this, the repressive behavior of the EK region during in vivo splicing is readily apparent. Deletion of the EK domain from SRrp86 resulted in a 6-fold increase in exon 4 inclusion. Similarly, addition of the EK domain to SRp75 clearly eliminated its ability to increase exon 4 inclusion.

**The EK Domain Blocks Constitutive Splicing**—Because the EK domain functioned like a repressor in alternative splicing, we next wanted to know whether it could also repress constitutive splicing. For these experiments, an adenovirus-derived transcript (AdeML) was used as a substrate for splicing in limiting amounts of nuclear extract such that addition of SR proteins was required to activate splicing (Fig. 6). As shown, addition of either purified SR proteins or recombinant SRp75 greatly increased splicing efficiency, leading to efficient accumulation of spliced mRNA. Consistent with previous results, SRrp86 was unable to rescue splicing in such extracts (44). To test the effect of the EK domain on the rescue of splicing, the SRp75 + EK and SRp75ΔEK chimeric proteins were added to identical splicing reactions. As shown, removal of the EK region from SRp75 clearly rescued splicing, whereas insertion of the EK domain into SRp75 blocked its ability to rescue splicing. Thus, as with alternative splicing, the EK domain can function to repress the activity of proteins to rescue constitutive splicing.

**Incubation of Extracts with the EK Domain Blocks Splicing**—To determine whether the EK domain could inhibit splicing on its own, a histidine-tagged version was purified from E. coli and added to splicing reactions in HeLa nuclear extracts. As shown in Fig. 7, addition of increasing amounts of recombinant EK peptide inhibited splicing. To exclude the possibility that such inhibition could have been nonspecific, control bacterial protein preparations were generated using empty-vector-transformed E. coli and identical purification of lysates over Ni-NTA agarose beads. Furthermore, the histidine-tagged EK
peptide was subjected to heat denaturation. In both cases, neither the control extract nor the boiled peptide was able to inhibit splicing to the same extent as the native peptide. Inhibition was only observed at higher concentrations, and the decrease in splicing efficiency was fairly modest (Fig. 7). In contrast, the EK peptide showed a more robust dose-dependent inhibition of splicing. Taken together, these results demonstrate that the inhibition of splicing observed with the EK peptide is specific and implies that the EK domain binds to one or more splicing factors in nuclear extract and titrates their ability to activate splicing.

To understand whether the inhibition of splicing by the EK domain occurs during early or late spliceosome formation, splicing reactions identical to those shown in Fig. 6 with limiting amounts of nuclear extract were performed followed by native gel electrophoresis. After 5 or 10 min of incubation in the presence of purified SR proteins or the indicated chimeric proteins, spliceosome complexes were analyzed (Fig. 8). Splicing reactions in unsupplemented extract under these conditions generated complexes that correspond to the H, A, and B pre-spliceosome complexes. It is possible that the B complex co-migrates with catalytic spliceosomes (C complexes) but since no spliced product can be detected at these early time points (data not shown), it is likely that most or all of the slowest migrating band consists of B complexes. Regardless, addition of purified SR proteins or recombinant SRp75 clearly increased the formation of these complexes (Fig. 8A). In contrast, addition of the EK domain to SRp75 inhibited its ability to promote spliceosome assembly. Therefore, it appears that the EK domain acts to repress early stages of spliceosome assembly. Deletion of the EK domain from SRrp86 also resulted in increased levels of spliceosome complex formation, although it was somewhat surprising that SRrp86 slightly increased such levels by itself because it cannot rescue splicing under these conditions (Fig. 6). Nevertheless, deletion of the EK domain from SRrp86 increased spliceosome complex formation over that seen with the parental protein, supporting the idea that the EK domain inhibits splicing at early stages of spliceosome assembly (Fig. 8B).

**DISCUSSION**

In this report, we used domain-swap experiments to analyze the functional properties of SRrp86, a regulator of SR proteins. Despite the fact that the two RS domains from SRrp86 are atypical, chimeric constructs with these domains joined to the RRMs from ASP/SF2 or SRp75 could still regulate alternative splice-site selection. However, when the converse exchange was created, fusing the RS domains from ASP/SF2 or SRp75 to the RRM from SRrp86, we discovered that the RS domains are functionally distinct; only the RS domains from SRp75 were able to activate splicing and alter splice-site selection. For the EK domain, deletion and chimeric constructs suggest that it acts to inhibit splicing and splice-site selection, perhaps by titrating the activity of required splicing factors.

**Domain Analysis of SRrp86**—Even though the parental SRrp86 protein by itself could not activate splicing or alter splice-site selection, its RR and RS domains were both capable of such activity in heterologous settings, indicating that the precise function of the parental protein is caused by its unique combination of domains. This combination allows SRrp86 to function as a modulator of splicing, apparently through direct interaction with other SR proteins (44, 48). Recent protein-protein analyses and mass spectrometry identification of associated proteins supports the idea that SRrp86 preferentially interacts with a distinct subset of SR proteins as well as other splicing-related factors.

The fact that incubation of splicing extracts with purified EK polypeptide inhibited splicing supports the idea that it functions by interacting with other splicing factors. The inhibitory effects of the EK peptide could be explained by two possible protein-protein interaction mechanisms. First, the EK domain might recruit inhibitory factors to block splicing, or second, the EK domain might bind splicing factors and form dead-end complexes. The latter effect may be due to the fact that, in contrast to RS domains, the EK domain is unable to undergo regulated negative charge removal that may be crucial for subsequent spliceosomal rearrangements. We are currently working to identify which factors bind the EK domain.

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