The STAR/GSG Family Protein rSLM-2 Regulates the Selection of Alternative Splice Sites*

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We identified the rat Sam68-like mammalian protein (rSLM-2), a member of the STAR (signal transduction
and activation of RNA) protein family as a novel splicing regulatory protein. Using the yeast two-hybrid system,
coimmunoprecipitations, and pull-down assays, we demonstrate that rSLM-2 interacts with various pro-
teins involved in the regulation of alternative splicing, among them the serine/arginine-rich protein SRp30c,
the splicing-associated factor YT521-B and the scaffold attachment factor B. rSLM-2 can influence the splicing
pattern of the CD44v5, human transformer-2β and tau minigenes in cotransfection experiments. This effect
can be reversed by rSLM-2-interacting proteins. Employing rSLM-2 deletion variants, gel mobility shift as-
says, and linker scan mutations of the CD44 minigenes, we show that the rSLM-2-dependent inclusion of exon v5
of the CD44 pre-mRNA is dependent on a short purine-rich sequence. Because the related protein of rSLM-2,
Sam68, is believed to play a role as an adapter protein during signal transduction, we postulate that rSLM-2 is a
link between signal transduction pathways and pre-mRNA processing.

Prior to export to the cytosol, pre-mRNA generated from most eukaryotic genes undergoes maturation processes such as
splicing, in which intronic sequences are removed and exonic sequences are rejoined, as well as polyadenylation and 5'-end
capping. There is increasing evidence that transcription, pre-mRNA processing, and RNA transport are coupled in a
highly coordinated manner (1–3). Recent results indicate that a direct interaction among RNA polymerase II, transcription,
capping, splicing, and polyadenylation factors (3–6). These complexes are possibly attached to chromatin, for example by
the scaffold attachment factor B (SAF-B)

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‡ The abbreviations used are: SAF-B, scaffold attachment factor B; EGFP, enhanced green fluorescent protein; GST, glutathione S-transfer-
ase; HEG, human embryonic kidney; htra2-β, human transformer-2-beta; hnRNP, heterogeneous nuclear ribonucleoprotein; KH domain,
hnRNP K homology domain; mAb, monoclonal antibody; Sam68, Src-related protein of rSLM-2, associated in mitosis; SLM, Sam68-like mammalian protein; SR pro-
tein, serine/arginine-rich protein; STAR, signal transduction and activation of RNA.
teins involved in signal transduction such as phospholipase Cγ or p85 phosphatidylinositol 3-kinase, this protein has been proposed to play a role as an adapter protein in fibroblast and lymphocyte signaling (32–35). In addition, there is increasing evidence that Sam68 is important for cell cycle progression (36–38). Recently, two nuclear Sam68-like mammalian proteins (SLM-1 and SLM-2, also called T-STAR/etoile/Salp) have been described (39–41). However, the exact function of Sam68 and its relatives in the nucleus still remains elusive.

Here, we demonstrate for the first time the involvement of the Sam68-like mammalian protein rSLM-2 in the regulation of alternative splicing. This protein interacts with splicing regulatory proteins in vivo and in vitro and influences the splice site selection of three different minigenes in a concentration-dependent manner. Using a CD44 minigene, we show that the rSLM-2-dependent inclusion of exon v5 depends on a purine-rich sequence to which rSLM-2 binds in vitro.

**MATERIALS AND METHODS**

**Two-hybrid Screening and Cloning—**A yeast two-hybrid screen and interaction experiments were performed as described (26, 42). Using rSAF-B as bait in pGBT9, 200,000 colonies of a rat brain embryonic day E16 library (Stratagene, pAdGal4-cDNA as prey) were screened. The DNA of six lacZ-positive clones able to grow on selective medium containing 10 mM 3-aminotriazole was sequenced as described (26). An amino-terminal FLAG tag was introduced to the full-length form of rSLM-2 by polymerase chain reaction followed by subcloning into pDNA1.

**Antiserum Production and Purification—**Peptides specific for rSLM-2 VVTGKSLTRGTVCG and PRAGVPPTGYRPGC were coupled to keyhole limpet hemocyanin and used to immunize rabbits. After 121 days, serum was purified by affinity chromatography, employing a mixture of recombinant GST-rSLM-2 and the two peptides following the manufacturers instructions (Pierce). Dilution for Western blot was described (26). The following antibodies were used: anti-rSLM-2 (1:2,000) (25), PY20 (1:10,000), anti-Src (1:1,000 and for immunohistochemistry 1:100). Preabsorption of 10 pmol of the 32P-labeled RNA oligonucleotides for 15 min at 30 °C. The indicated amounts of recombinant GST-rSLM-2 were incubated with 30 pmol of the reactions were incubated with 1 µg of GST or GST-rSLM-2 coupled to glutathione-Sepharose 4B (Amersham Pharmacia Biotech) in the presence of 200 µl of HNTG buffer and 0.1% Triton X-100 (43) for 2 h at 4 °C. Washing and detection were as described (43).

**RNA Gel Shift Assay—**RNA gel shift assays were performed as described (24). End labeling of the RNA oligonucleotides was performed with T4 polynucleotide kinase (New England Biolabs) and [γ-32P]ATP followed by purification using the nucleotide removal kit (Qiagen). Indicated amounts of recombinant GST-rSLM-2 were incubated with 30 pmol of the 32P-labeled RNA oligonucleotides for 15 min at 30 °C. The oligonucleotide sequences were: r-rich, GAGGGAGAAAGAGGAGGAGAA; y-rich, CUCUCUUCCUCCUCCUCUCUCUCUCUU; v5, AGUACUGCGAAGGAGGAGGAGCGCAUCAGCAAGGCAA; v6s9, AGUACUGCGAAGGAGGAGGAGCGCAGCAUCAGCAAGGCAA.

**In Vivo Splicing Assays—**These assays were essentially performed as described (59) employing the CAD4v5 (44), the htr2α-h1 (23), tau minigenes containing exon 2 or exon 3 (45), or clathrin light chain B minigenes (24, 46). Transfection of the CAD4v5 and clathrin light chain B minigenes in HEK293 cells or the htr2α-h1 and tau minigenes were analyzed in HN10 cells and COS cells, respectively. For the CAD4v5 and clathrin light chain B minigenes, polymerase chain reaction conditions were as described (24, 44). For the htr2α-h1 minigene, 20-s denaturation at 94 °C, 20-s annealing at 65 °C, and 40-s extension at 72 °C for 33 cycles were used followed by a final extension at 72 °C for 20 min.

For the tau minigenes, polymerase chain reaction was carried out for 22 cycles with 94 °C for 1 min, annealing at 65 °C for 1 min, and extension at 72 °C for 1 min. The resulting splicing pattern was quantified using the Herolab EASY system.

**RESULTS**

**Molecular Cloning and Sequence of rSLM-2—**SAF-B was initially cloned because of its strong interaction with scaffold/matrix attachment regions (47). Recently, we described the interaction of SAF-B with RNA polymerase II as well as with splicing factors (7). In addition, SAF-B changed the adenovirus E1A alternative splicing pattern in a concentration-dependent manner (7).

To find new proteins involved in the regulation of alternative pre-mRNA splicing, we used the yeast two-hybrid system to screen an embryonic (E16) rat brain library with rSAF-B (7) as an interacting partner. From about 200,000 colonies screened, six lacZ-positive clones were able to grow in the presence of 5 mM 3-aminotriazole. Two clones contained an open reading frame bearing 67% homology to human Sam68 (27). The predicted protein shares the typical domain structure of the members of the STARB family (28) and shows 96% sequence identity to the recently identified mouse protein rSLM-2/Salp/SLM-2-like mammalian protein SLM-2 (39–41). It was therefore named rSLM-2. The protein has a maxi-KH homology RNA binding motif (shadowed box, Fig. 1). Similar to Sam68, rSLM-2 has a central region containing multiple arginine/glycine (RG) dipeptides, followed by a stretch of six prolines which matches the class I consensus SH3 domain binding site (48). The carboxyl-
The terminal part of rSLM-2 is rich in tyrosine residues and represents a potential SH2 domain binding site (48, 49).

Identification of Novel Interactors of rSLM-2 Using the Yeast Two-hybrid Screen—To obtain information on the function of rSLM-2, we performed yeast two-hybrid screens with rSLM-2 as an interacting partner in an embryonic rat brain library. It has been shown previously that SLM-2 interacts with the highly homologous protein Sam68 (39), with itself as well as with hnRNP G family members (40). Our two-hybrid results with rSLM-2 confirmed these interactions because we isolated the rat homologs of Sam68 and hnRNP G. In addition, among the lacZ-positive yeast colonies that were able to grow on selective medium containing 5 mM 3-aminotriazole, we identified four novel interactors of rSLM-2: rSAF-B, which has initially been used to clone rSLM-2; the novel splicing-associated protein YT521-B (26, 50); hnRNP L (51); and the SR-protein SRp30c (52) (Fig. 2A). In addition, we tested its interaction with another KH domain containing RNA-binding protein, SF1 (53). Although Sam68 was shown to bind to other KH domain-containing proteins such as Bicaudal C or Grp33 (54), rSLM-2 did not interact with the splicing factor SF1, indicating the specificity of the observed interactions.
hnRNP L, YT521-B, SRp30c, and htra2-b rSLM-2 and radiolabeled hnRNPs or SR proteins. rSAF-B, hnRNP L, YT521-B, SRp30c, and htra2-b were translated in vitro in the presence of [35S]Met using a coupled transcription/translation reaction in reticulocyte lysates. These proteins were incubated with GST-rSLM-2 coupled to glutathione- Sepharose 4B. The input of radiolabeled proteins is shown in lanes 1–5. Retained proteins were analyzed on a 15% SDS-PAGE (lanes 6–10). GST alone was used as a negative control (lanes 11–15).

These novel two-hybrid interactions were tested subsequently using coimmunoprecipitations (Fig. 2B). We generated a specific antibody against rSLM-2 which did not cross-react with the related proteins Sam68 or rSLM-1. To exclude nucleic acid-mediated interactions, benzonase was present in all experiments. In addition, the RNA-binding protein SF1 (53) was used as a negative control (Fig. 2B, bottom row). We expressed EGFP-tagged proteins in HEK293 cells and precipitated them using a monoclonal anti-GFP antibody. Overexpressed EGFP was used as a negative control. The efficiency of the immunoprecipitation was analyzed in Western blots with a polyclonal anti-GFP antibody (Fig. 2B, left column). The coimmunoprecipitating proteins and cell lysates were analyzed by Western blot (Fig. 2B, middle column) using the appropriate antibody (right column). Using this method, we found endogenous hSAF-B to bind to rSLM-2 (Fig. 2B, top row). In agreement with previous data (39, 40) and our data obtained in yeast, cotransfected hnRNP G and endogenous Sam68 interacted with rSLM-2 (data not shown). Finally, we confirmed the interaction of rSLM-2 with hnRNP L (second row), with the splicing associated protein YT521-B (third row), and the interaction of rSLM-2 with the splicing factor SRp30c (fourth row).

In summary, EGFP-rSLM-2 was able to homomultimerize and to bind to a subset of splicing and splicing-associated factors in the yeast two-hybrid system as well as in coimmunoprecipitations.

rSLM-2 Interacts with hnRNPs and SR Proteins in Vitro—To confirm further the observed interactions of rSLM-2, we performed pull-down experiments using in vitro translated rSAF-B, hnRNP L, YT521-B, SRp30c, and htra2-b (Fig. 3, lanes 1–5), as well as recombinant GST-rSLM-2 protein (Fig. 3, lanes 6–10). As shown in lanes 6–9 in Fig. 3, the hnRNPs rSAF-B and hnRNP L, as well as the splicing-associated protein YT521-B and the SR protein SRp30c, bound directly to rSLM-2. In contrast, htra2-b did not bind to GST-rSLM-2 (Fig. 3, lane 10), which is in agreement with our data obtained in yeast and demonstrates the specificity of the interactions.

rSLM-2 Forms a Complex with SR Proteins in Vivo—It has been shown previously that the rSLM-2 interactor SAF-B binds to htra2-b, SRp30c, SF2/ASF, and hnRNP A1 and can change alternative splice sites in vivo (7, 55). Therefore, we asked whether rSLM-2 is present in a complex with splicing factors in vivo. Screening of several cell lines revealed that SLM-2 is expressed in HN10 cells. These cells are derived from hippocampal neurons (56) that express SLM-2 (Fig. 4A, Lysate).

We performed an immunoprecipitation in HN10 cell lysates with the pan SR antibody mAb104 (57) in the presence of benzonase and found rSLM-2 to be present in this complex (Fig. 4A, IP). One-third of the corresponding immunoprecipitates were analyzed with the mAb104 antibody (Fig. 4B) to verify the successful immunoprecipitation. Furthermore, mAb104 did not cross-react with rSLM-2, even when we overexpressed EGFP-rSLM-2 (Fig. 4C). We conclude that endogenous rSLM-2 forms an in vivo complex with factors implicated in pre-mRNA splicing.

rSLM-2 Can Change Alternative Splicing Patterns—The association of rSLM-2 with splicing factors as well with the splicing-associated protein YT521-B and hnRNPs points to a role in pre-mRNA processing. Therefore, we asked whether rSLM-2 could modulate splice site selection in different cell lines in a concentration-dependent manner, analogous to several proteins involved in splicing, such as SR proteins (14, 58), hnRNPs (58), SAF-B (7), and YT521-B (26). To investigate this possibility, we employed a CD44 reporter gene, which contains the alternative exon v5 (44). This minigene was transfected with increasing amounts of EGFP-rSLM-2 in HEK293 cells. Vector DNA (pEGFP) was added to ensure that comparable amounts of DNA were transfected in each experiment (59). Increasing the amount of transfected pEGFP-rSLM-2 resulted in an increased incorporation of exon v5, as shown in Fig. 5A, suggesting a rSLM-2 concentration-dependent modulation of splice site selection. Western blots with lysates from the transfections confirmed an increase in EGFP-rSLM-2 expression (data not shown).

Next, we determined the role of various rSLM-2 domains and tested deletion variants for their influence on splice site selection. As shown in Fig. 5B, deletion of either the RG-rich region and the potential SH2 and SH3 binding domains (rSLM-2Δ2), the tyrosine-rich SH2 domain binding site (rSLM-2Δ3), or the KH domain (rSLM-2Δ4) abolished the influence on splice site selection. All deletion variants were expressed at similar levels in the transfected cells (Fig. 5C). We conclude that the ability of rSLM-2 to bind to RNA is necessary for splice site selection. However, in addition to RNA binding, the potential protein interaction sites are also necessary for the splice site switch. This suggests that rSLM-2 acts in a complex with other proteins on pre-mRNA splicing.

We therefore wondered whether proteins binding to rSLM-2 can influence the regulation of alternative splicing of the CD44 minigene and analyzed the interactors SRp30c, hnRNP G, and SAF-B. First, we tested them separately with the CD44 minigene and found that SRp30c slightly repressed exon v5, whereas hnRNP G and SAF-B had no effect on exon v5 usage.

* O. Stoss, M. Olbrich, A. M. Hartmann, and S. Stamm, manuscript in preparation.
Then, we analyzed these interactors in the presence of rSLM-2 and found that all of them led to a significant decrease of the exon v5 stimulation by rSLM-2 (Fig. 5E). These data provide evidence that the rSLM-2-dependent inclusion of exon v5 can be antagonized by SRp30c, hnRNP G, and SAF-B.

The rSLM-2-dependent Exon Inclusion Depends on a Purine-rich Exonic Splicing Enhancer—Pull-down assays on immobilized RNA with HEK293 cell lysates containing overexpressed rSLM-2 showed that rSLM-2 selectively binds to purine-rich RNA (39).3 Previously, a purine-rich splice enhancer has been characterized in exon v5 of the CD44 gene using linker-scan mutations (44). Because the analysis of rSLM-2 deletion variants demonstrated the necessity of its RNA binding domain in splice site regulation (Fig. 5B), we were interested in determining the target sequence of the CD44 pre-mRNA. We compared the influence of rSLM-2 on three different CD44 minigene mutants ls8–ls10 (Fig. 6A). In the ls9 variant, the purine-rich enhancer sequence is replaced. Similar to the wild type minigene, EGFP-rSLM-2 was able to induce exon v5 inclusion with the ls8 and ls10 minigene constructs in HEK293 cells (Fig. 6B).

However, when 10 purine-rich nucleotides have been replaced (ls9), we detected a drastic decrease in the rSLM-2-dependent exon v5 inclusion rate (Fig. 6A). Instead of 80% (± 4.8%) exon v5 inclusion, we only observed 42% (± 5.1%) exon inclusion. With EGFP alone, the default splicing pattern was exon v5 skipping in each case.

To determine whether recombinant GST-rSLM-2 binds directly to CD44 exon v5 RNA, we performed gel mobility shift assays with several RNA oligomers. First, we determined that GST-rSLM-2 binds to the purine-rich oligonucleotide r-rich (Fig. 6D). With increasing amounts of rSLM-2, a super shift is visible which is probably due to the ability of rSLM-2 to multimerize (Fig. 6C). This is consistent with our two-hybrid and immunoprecipitation data and the observation that GST-rSLM-2 migrates as a complex of about 450 kDa upon gel filtration (data not shown). Next, we employed the oligonucleotide v5, which contains the regulatory sequence of the CD44 exon v5 and observed binding of rSLM-2 to it (Fig. 6D, lanes 1–3). Then we used an oligonucleotide (v5ls9) containing the mutation ls9, which abolished the effect of rSLM-2 on CD44 exon v5 regulation in vivo (Fig. 6B). As shown in Fig. 6D, lanes 4–6, no binding is observed with the v5Rls9 oligonucleotide. Furthermore, we observed binding to the purine-rich oligonucleotide

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3 O. Stoss, M. Olbrich, A. M. Hartmann, H. König, J. Memmott, A. Andreadis, and S. Stamm, unpublished results.
The locations of the 10-base pair linker scan mutations ls8, ls9, and ls10 within exon v5 are indicated (44). In the mutant ls9 the purine-rich sequence ATGAAGAGGA is replaced by CGACGCCTCG. Panel B, the effect of EGFP-rSLM-2 on three different linker scan mutations (ls8–10) in exon v5 of the CD44 minigene has been compared with the effect of EGFP. With the ls8 and ls10 minigenes, 2 μg of transfected EGFP-rSLM-2 increased exon v5 inclusion similar to the wild type minigene. In contrast, only a minor effect of EGFP-rSLM-2 was detected with the ls9 construct. HEK293 cells were used for transient transfections. Panel C, recombinant rSLM-2 binds to RNA in vitro. An increasing amount of GST-rSLM-2 was incubated with a 32P-labeled polypurine RNA oligonucleotide (r-rich) and separated on a nondenaturing gel. The RNA-rSLM-2 complex is indicated by a closed arrow, a larger complex observed at higher rSLM-2 concentration with an open arrow. Panel D, recombinant rSLM-2 binds the purine-rich exon v5 enhancer in vitro. 30 pmol of GST-rSLM-2 was incubated with the RNA oligonucleotides v5, v5ls9, r-rich, and y-rich. Each oligonucleotide was competed with the 20- and 40-fold excess of unlabeled v5 oligonucleotide (lanes 2 and 3; 5 and 6; 8 and 9; 11 and 12). Upon longer exposure, binding of the v5ls9 oligonucleotide is observed only in the absence of competitor. The dot indicates an unspecific complex seen with the y-rich oligonucleotide that is competed by excess of r-rich oligonucleotide. No shift is observed with GST (lanes 13–16) or without protein (lanes 17–20).
expression of rSLM-2 stimulates exon 3 usage, whereas EGFP alone has no effect (Fig. 7B). Interestingly, a sequence stretch in exon 2 of htra2-β and a sequence stretch in exon 3 of the tau transcript show a significant homology to the purine-rich sequence of exon v5 of the CD44 minigene, which is necessary for the rSLM-2-dependent exon inclusion.

Finally, overexpression of rSLM-2 did not stimulate inclusion of the pyrimidine-rich neuron-specific exon EN of the clathrin light chain B transcript (data not shown) and did not repress exon EN usage when this exon is activated by improving its 5'-splice site (Fig. 7C). In contrast, htra2-β1 can regulate splice site usage of this construct (Fig. 7C), which emphasizes the specificity of rSLM-2-mediated splice site selection. Taken together, this indicates that rSLM-2 can change the alternative splicing pattern of specific substrates in a concentration-dependent manner by binding to purine-rich sequences.

**DISCUSSION**

rSLM-2 Interacts with Proteins Involved in Splice Site Selection—SAF-B has been shown to associate with the splicing factors SRp30c, ASF/SF2, and htra2-β1 as well as with the carboxyl-terminal domain of the largest subunit of RNA polymerase II, which has also been shown to stimulate pre-mRNA splicing (7, 63). In addition, SAF-B binds to matrix attachment regions and stimulates the generation of the 10S E1A transcript in a concentration-dependent manner (7).

To identify novel proteins involved in mRNA processing, we performed a yeast two-hybrid screen using SAF-B as a bait. This led to the identification of rSLM-2 as a novel interactor of SAF-B.

rSLM-2 and its close relatives Sam68 and rSLM-1 are members of the STAR protein family (29), also called GSG protein family (30). These nuclear proteins combine a slightly modified
hnRNP K homology RNA binding domain, called maxi-KH domain, with binding sites for proteins containing phosphotyrosine or proline binding domains like SH2, SH3, or WW domains. To investigate the function of rSLM-2, we searched for novel interactors using yeast two-hybrid screens and further confirmed these interactions with coimmunoprecipitations and in vitro interaction assays using recombinant rSLM-2 protein. Interestingly, all of the rSLM-2-interacting proteins found within these three independent systems are implicated in the regulation of alternative splicing. In addition to SAF-B, which has initially been used to isolate rSLM-2, the splicing-associated protein YT521-B has been found to interact with rSLM-2. Using the SRp20 and htra2-β minigenes, we recently demonstrated the influence of YT521-B on alternative splicing (26). Like rSLM-2, YT521-B interacts with Sam68 and is tyrosine-phosphorylated upon overexpression of the tyrosine kinases Sre and Fyn (26). It remains to be determined whether rSLM-2 is tyrosine-phosphorylated by nuclear tyrosine kinases, such as SIK/BRK, which was shown to phosphorylate Sam68 (64, 65).

It is notable that rSLM-2, SAF-B, and YT521-B all interact with the splicing factor SRp30c. The physiological significance of this complex formation of rSLM-2 has been demonstrated by endogenous coimmunoprecipitation using the pan anti-SR protein antibody mAb104 (Fig. 4). However, in contrast to SAF-B and YT521-B, no direct binding could be detected between rSLM-2 and the splicing factors htra2-β1 (Figs. 2A and 3) or SF1 (Fig. 2). This demonstrates the specificity of the analyzed interactions, which have been carried out in the presence of benzamidine to avoid RNA-dependent protein interactions.

Finally, a growing number of hnRNP's have been characterized as molecular players in the regulation of alternative splicing, among them hnRNP A1 (58), hnRNP F, hnRNP H (15), hnRNP I (polyuridylinate tract binding) (16), hnRNP L (66), and a testis-specific member of the hnRNP G family, RNA-binding motif (RBM) (67, 68). Using a two-hybrid screen with RBM as a bait, Venables et al. were previously able to isolate the human homologue of rSLM-2, T-STAR (40). Now we provide evidence that rSLM-2 also interacts with the ubiquitously expressed hnRNP G and with hnRNP L.

rSLM-2 Regulates Alternative Splice Site Selection—The binding properties of rSLM-2 indicate a role in pre-mRNA processing. We tested this hypothesis by performing transient transfection assays with several reporter minigenes that contained purine-rich sequences in their alternative exons. As a control, a pyrimidine-rich exon of clathrin light chain B was employed. We found that rSLM-2 changes the splicing patterns of several alternatively spliced exons in a concentration-dependent manner. It induced inclusion of exon v5 of CD44 (44) (Fig. 5A), of exon 3 of neurofilament tau (Fig. 7B) (69), but caused exon II skipping of the htra2-β exon II (23) (Fig. 7A). The opposing effect of rSLM-2 on different minigenes is reminiscent of the effects of splicing factors on natural minigenes in vivo. For example, SF2/ASF promotes exon EN inclusion when tested with the clathrin light chain B minigene (58) but causes exon 4 skipping with the SRp20 minigene (70).

Our deletion analysis showed that binding of rSLM-2 to RNA is not sufficient to influence splice site selection. The deletion mutant lacking a functional KH domain rSLM-2(ΔK) is exclusively nuclear (data not shown). In contrast, mutants lacking parts of the carboxyl terminus (rSLM-2Δ2 and Δ3) are also present in the cytosol (data not shown). This indicates that potential protein binding sites such as the tyrosine-rich carboxyl terminus or the proline-rich region are also necessary for activity and proper localization of rSLM-2. Interestingly, the rSLM-2-interacting proteins SAF-B, hnRNP G, and SRp30c inhibited exon v5 inclusion mediated by rSLM-2 (Fig. 5D), although these three interactors alone exerted only a minor or no effect on the CD44 minigene. Because SAF-B, hnRNP G, and SRp30c are ubiquitously expressed but rSLM-2 is predominantly expressed in muscle, brain, and testis (40 and data not shown), this suggests that cell type-specific combinations of rSLM-2 and some of its interactors may contribute to different splicing patterns in different cell types. This supports the model that different concentrations of antagonizing factors govern splice site selection.

Binding of rSLM-2 to a purine-rich RNA sequence is necessary to regulate splice site selection because either deleting its RNA binding domain or changing the purine-rich sequence abolishes an effect on usage of exon v5 (Figs. 5B and 6B).

Because rSLM-2 binds directly to purine-rich RNA (Fig. 6, C and D), it is likely that it regulates splice site selection through interaction with purine-rich sequences in vivo. The in vitro binding of rSLM-2 to exon v5 RNA, but not to the mutant v5ls9 (Fig. 6D) is in complete agreement with the regulation of CD44 exon v5, but not CD44 exon v5ls9 by rSLM-2 (Fig. 6B). In addition, rSLM-2 cannot regulate the pyrimidine-rich exon EN of clathrin light chain B (Fig. 7C), even when this exon is activated by a 5'-splice site improvement. A sequence comparison between the regulated exons of CD44, htra2-β1 and tau (Fig. 8) also suggests a purine-rich motif as the likely site of action.

Our data provide for the first time evidence for a role of a STAR protein in the regulation of alternative splicing. In addition, there is increasing evidence that the STAR protein Sam68 is also involved in alternative splice site selection. First, Sam68 has been found to cross-link to an intronic regulatory RNA sequence of the tropomyosin pre-mRNA (71). Second, Bedford et al. recently demonstrated the binding of Sam68 to the spliceosome-associated protein FBP21 (72). Finally, like rSLM-2, Sam68 interacts with the testis-specific splicing factor RBM (67). However, a direct effect of Sam68 on alternative splice site selection remains to be shown.

In addition to the role of Sam68 in viral replication (73, 74), two possible functions of rSLM-2 and Sam68 have been investigated to date. First, there is evidence that rSLM-2 and Sam68 are important for cell cycle progression (27, 28, 36, 41), but a direct molecular link is still missing. We speculate that STAR proteins influence the cell cycle by regulating mRNAs necessary for its progression.

Furthermore, Sam68 has been proposed to act as an adapter protein within signal transduction pathways. Upon T cell or insulin receptor stimulation, Sam68 is tyrosine-phosphorylated (33–35). Phosphorylation changes its binding affinities to phospholipase Cγ1, to the regulatory p85 subunit of phosphatidylinositol 3-kinase (32), to itself, and to RNA (54, 75). The p85 phosphatidylinositol 3-kinase also binds to the human homolog of rSLM-2 (41). There are numerous examples for the regulation of alternative splicing by extracellular stimuli (for review, see Ref. 76). Because this list of stimuli which includes insulin (77), nerve growth factor (78), cytokines (79), or neuronal activity (25) is rapidly growing, we are now investigating whether rSLM-2 and similar adapter proteins are part of a signal transduction cascade from receptors toward the spliceosome.

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