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p59^{fyn}-mediated phosphorylation regulates the activity of the tissue-specific splicing factor rSLM-1☆

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The Sam68-like mammalian protein SLM-1 is a member of the STAR protein family and is related to SAM68 and SLM-2. Here, we demonstrate that rSLM-1 interacts with itself, scaffold-attachment factor B, YT521-B, SAM68, rSLM-2, SRp30c, and hnRNP G. rSLM-1 regulates splice site selection in vivo via a purine-rich enhancer. In contrast to the widely expressed SAM68 and rSLM-2 proteins, rSLM-1 is found primarily in brain and, to a much smaller degree, in testis. In the brain, rSLM-1 and rSLM-2 are predominantly expressed in different neurons. In the hippocampal formation, rSLM-1 is present only in the dentate gyrus, whereas rSLM-2 is found in the pyramidal cells of the CA1, CA3, and CA4 regions. rSLM-1, but not rSLM-2, is phosphorylated by p59^{fyn}. p59^{fyn}-mediated phosphorylation abolishes the ability of rSLM-1 to regulate splice site selection, but has no effect on rSLM-2 activity. This suggests that rSLM-1-positive cells could respond with a change of their splicing pattern to p59^{fyn} activation, whereas rSLM-2-positive cells would not be affected. Together, our data indicate that rSLM-1 is a tissue-specific splicing factor whose activity is regulated by tyrosine phosphorylation signals emanating from p59^{fyn}.

Introduction

Alternative splicing of pre-mRNA transcripts is a common mechanism to generate protein isoforms from a single gene. A detailed analysis of chromosome 22 (Lander et al., 2001) and genome-wide EST-based comparisons show that 47–59% (Modrek et al., 2001) of human genes are alternatively spliced.

Since splice sites can be only described by degenerate consensus sequences, auxiliary sequences in the exon, or its vicinity are necessary to define an exon (Black, 2003). These degenerate RNA-elements interact with proteins that can be subgrouped into SR proteins and hnRNPs (Dreyfuss et al., 2002; Graveley, 2000). SR proteins bind RNA and contain carboxy terminal serine-arginine dipeptide clusters. Using this RS domain, they directly interact with and recruit components of the general splicing machinery to splice sites (Cartegni et al., 2002). HnRNPs often block this recruitment, which antagonizes the action of SR-proteins (Zhu et al., 2001). As a result, the relative concentrations of protein factors can regulate alternative exon usage (Roberts and Smith, 2002; Smith and Valcarcel, 2000). Furthermore, tissue-specific hnRNPs, such as NOVA-1, can regulate tissue-specific splicing events, such as the inclusion of the glycine receptor alpha2 exon 3A (Jensen et al., 2000; Ule et al., 2003).

Database analysis shows that the number of tissue-specific alternative splice forms is highest in brain (Stamm et al., 2000; Xu et al., 2002), which underlines the importance of this mechanism for the nervous system (reviewed in Grabowski and Black, 2001).

The selection of alternative splice sites can be modulated in response to cellular signals (Stamm, 2002). Signal transduction and activation of RNA (STAR), also called GSG (GRP33, SAM68, GLD-1)) proteins are a family of nuclear RNA-binding proteins that share an extended hnRNP K homology domain (KH domain) and C-terminal sequences typically involved in signal transduction. Examples of this family are Sam68 (Wong et al., 1992) and the related Sam68-like mammalian proteins SLM-1 and SLM-2 which share a KH-RNA-binding domain and proline- and tyrosine-rich stretches (Di Fruscio et al., 1999). SAM68 is the best characterized member of this family. It interacts with src kinases, (Richard et al., 1995), the insulin receptor (Sanchez-Margalet and Najib, 1999), and is a target of ERK kinases (Matter et al., 2002). SAM68 plays a role in alternative splicing (Matter et al., 2002), cell cycle regulation (Barlat et al., 1996; Taylor et al., 1995) and RNA export (Reddy et al., 2000). Its function is influenced by ERK-mediated threonine phosphorylation (Matter et al., 2002) and Sik/BRK-mediated tyrosine phosphorylation (Coyle et al., 2003).

Abbreviations: 3′ UTR, 3′ untranslated region; AT, 3-amino-triazole; DAPI, 4,6 Diamidino-2-phenylindole; EGFP, enhanced green fluorescent protein; EST, expressed sequence tag; htra2-beta, human transformer-2-beta; KH domain, hnRNP K homology domain; PBS, phosphate buffered saline; PCR, polymerase chain reaction; RRM, RNA recognition motif; Sam68, Src associated in mitosis; SDS, sodium dodecylsulfate; SR proteins, serine/arginine-rich proteins; STAR, signal transduction and activation of RNA.

☆ The sequences have been deposited in Genbank with the accession numbers: AF305618 and AF305619.

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Here, we demonstrate that the rat homologue of SLM-1 is a tissue-specific splicing factor, expressed only in testis and brain, where it is specific for a subset of neurons that do not express rSLM-2. In contrast to rSLM-2, rSLM-1 is phosphorylated by $p59^{fyn}$, which modulates the ability of rSLM-1 to regulate splice site selection.

Results

Two related sequences of SAM68 exist in rat

Using SAF-B (Nayler et al., 1998; Weighardt et al., 1999) as an interacting protein in a yeast two hybrid screen, we recently isolated

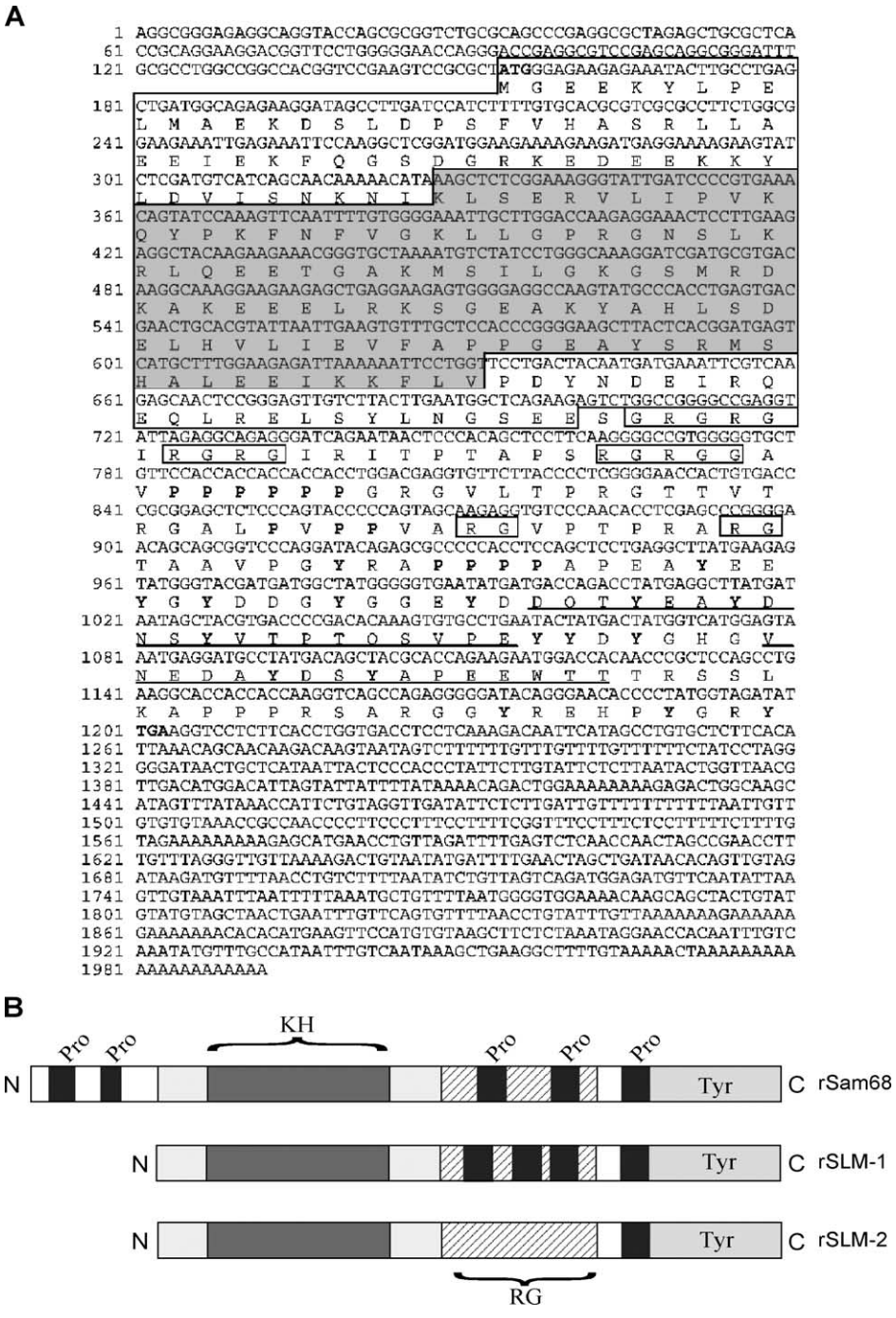


Fig. 1. Structure of rSLM-1. (A) cDNA and protein-sequence of rSLM-1. The cDNA sequence of rat SLM-1 is shown. Start and stop codons are shown in bold. The protein sequence is shown underneath the cDNA sequence. The KH RNA binding domain is shown as a shadowed box and is flanked by the QUA1 and QUA2 regions, indicated as open boxes. The arginine and glycine dipeptides clustered in the central part of the protein are boxed. Tyrosine residues in the carboxy terminal part of the protein are indicated in bold. The sequences of the peptides used to generate the antiserum against rSLM-1 are underlined. (B) Domain structure comparison of rSam68, rSLM-1 and rSLM-2. Pro: proline-rich regions; KH: hnRNP K homology domain; RG: arginine/glycine-rich region; Tyr: tyrosine-rich region.

the Sam68-like mammalian protein rSLM-2 (Stoss et al., 2001) from a rat brain cDNA library. In addition, we isolated cDNAs bearing high homology to the previously reported SLM-1 (Di Fruscio et al., 1999) and Sam68 (Richard et al., 1995) that we named rSLM-1 and rSAM68, respectively. rSam68, rSLM-1, and rSLM-2 are members of the signal transduction and activation of RNA (STAR) protein family (Vernet and Artzt, 1997), also called GSG family (Grp33, Sam68, Gls-1) (Jones and Schedl, 1995; Chen et al., 1999). The sequence of rSLM-1 is shown in Fig. 1A. Similar to rSam68 and rSLM-2, the protein has a maxi-KH RNA-binding domain (gray box), several arginine-glycine dipeptides and a tyrosine-rich carboxy terminus. The three highly related proteins, rSam68, rSLM-1, and rSLM-2, differ by the numbers of proline-rich stretches (Fig. 1B). The extended N-terminus of rSAM68 contains several ERK phosphorylation sites (Matter et al., 2002). We conclude that both in rat and human, three highly related cDNAs exist that share a similar, but not identical domain structure.

rSLM-1 interacts with proteins that function in splice site selection

We previously demonstrated that rSLM-2 acts as a splicing regulatory protein (Stoss et al., 2001). We assumed a similar function for rSLM-1 and tested proteins involved in splice site selection for their interaction with rSLM-1 in yeast. As shown in Fig. 2A, rSLM-1 multimerises with itself and the related proteins rSLM-2 and rSAM68. However, no interaction was seen between rSLM-1 and the more distantly related STAR protein SF1 (Rain et al., 1998). We found interaction of rSLM-1 with the hnRNPs SAF-B and hnRNP G (Nayler et al., 1998; Soulard et al., 1993; Weighardt et al., 1999). The only SR-protein binding to rSLM-1 was SRp30c (Screaton et al., 1995), whereas all other SR-proteins tested (ASF/SF2, Fig. 2A, and SC35, SRp40, SRp55, SRp75, data not shown) did not interact with rSLM-1. Finally, rSLM-1 interacts with YT521-B, a nuclear protein implicated in splice site selection (Hartmann et al., 1999; Nayler et al., 2000; Stoilov et al., 2002; Rafalska et al., 2004). Exhaustive two-hybrid screens with rSLM-1 in libraries from rat embryonic brain E15, postnatal brain P5, and adult brain did not reveal any other interactions.

To test whether the interactions observed in yeast were caused by direct protein–protein interaction, we performed GST-pull down assays. Recombinant GST-rSLM-1 was produced in bacteria and incubated with in vitro translated, radioactively labeled interacting proteins in the presence of glutathione sepharose. Confirming our results in yeast, the splicing regulatory proteins YT521-B, hnRNP G, SAF-B, and SRp30c bound to recombinant GST-rSLM-1 whereas the SR protein ASF/SF2 and hnRNP L failed to bind. None of the proteins bound to recombinant GST (Fig. 2B).

To determine whether the observed interactions could take place in eukaryotic cells, we employed coimmunoprecipitation experiments. EGFP-rSLM-1 was transiently expressed in HEK293 cells and immunoprecipitated with an anti-GFP antibody. Using anti-SAF-B and anti-YT521-B antisera, the corresponding endogenous interacting proteins could be identified in the immunoprecipitates (Fig. 2C). To test the interaction of rSLM-1 with SRp30c and hnRNP G, we coexpressed EGFP-tagged SRp30c and hnRNP G, FLAG-rSLM-1 in HEK293 cells. The multimerization of rSLM-1 was determined by cotransfecting EGFP-rSLM-1 and FLAG-rSLM-1. The EGFP-tagged proteins were immunoprecipitated and coprecipitated FLAG-rSLM-1 was identified using the anti-rSLM-1 antibody. All coimmunoprecipitations were performed in the presence of benzonase (Sigma) to avoid RNA- or DNA-mediated interactions. Using this technique, we could confirm binding of rSLM-1 to SRp30c, hnRNP G, and itself (Fig. 2C). Under these conditions, no interactions were observed with EGFP (Fig. 2C), SF1, or ASF/SF2 (data not shown). Probing of the immunoprecipitates with anti GFP confirmed the presence and integrity of the first precipitated protein (Fig. 2D). Finally, we raised peptide antisera against rSLM-1 and rSLM-2. The peptides were chosen in parts specific to rSLM-1 and rSLM-2 and no cross-reactivity between the STAR family members was observed (Fig. 2E) after affinity purification. The antisera detect proteins of the predicted size in lysates of Neuro2A cells (Fig. 2F). The signal from both the overexpressed (Fig. 2E) and endogenous (Fig. 2F) protein disappears after preabsorbtion, demonstrating the specificity of the antisera.

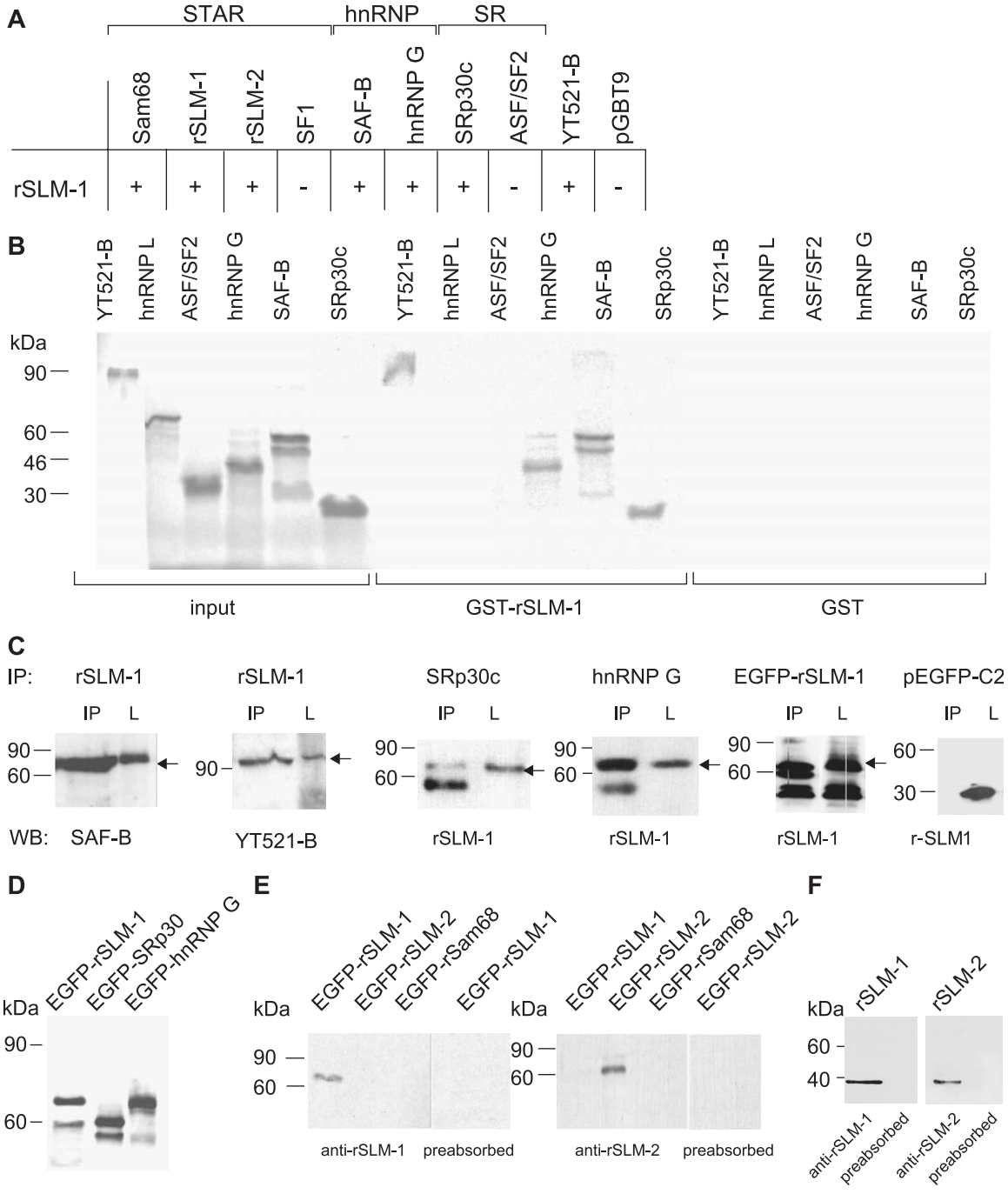
In conclusion, our data demonstrate that rSLM-1 directly interacts with itself, and several proteins functioning in RNA processing: SAF-B, hnRNP G, SRp30c, and YT521-B.

rSLM-1 changes alternative splicing patterns in a concentration-dependent manner

Previously, we demonstrated that rSLM-2 can change alternative splice site selection by binding to purine-rich enhancers

Fig. 2. Protein interactions of rSLM-1. (A) Yeast two hybrid interactions. The yeast strain HF7c was cotransfected with the cDNA of rSLM-1 fused to the Gal4-activation domain (“prey”) and one of the cDNAs to be tested fused to the Gal4-binding domain (“bait”). + and − indicates growth or absence of growth on selective medium lacking histidine in the presence of 5 mM 3-AT. None of the constructs showed self-activation and similar results were obtained when activation and binding domains were exchanged. The interacting proteins are grouped into STAR proteins, hnRNPs, and SR-proteins. pGBT9 is the parental, empty vector. (B) In vitro interaction assay. One microgram of recombinant GST or GST-rSLM-1 was incubated with 30,000 cpm of radiolabeled interacting proteins indicated in the presence of glutathione-Sepharose 4B. The inputs of the radioactive proteins are shown in the first six rows. YT521-B, hnRNP G, SAF-B, and SRp30c interacted with GST-rSLM-1 but not with GST. No interaction was seen for hnRNP L and ASF/SF2. (C) Coimmunoprecipitations. Proteins that were immunoprecipitated are shown on the top (IP), the antisera used to detect components of the immunoprecipitate are shown below the gels (WB). IP: immunoprecipitate, L: lysate, arrows point to the immunoprecipitates of the expected sizes. EGFP-rSLM-1 was expressed in HEK293 cells and precipitated using the anti-GFP antibody (Boehringer). Coimmunoprecipitated endogenous SAF-B or YT521-B (IP) and the corresponding lysates (L) were detected on Western blots using anti-SAF-B or anti-YT521-B antisera. To test the interaction of rSLM-1 with SRp30c and hnRNP G and itself, FLAG-rSLM-1 was coexpressed with EGFP-SRp30c or EGFP-hnRNP G. The EGFP constructs were immunoprecipitated using an anti-GFP antibody. The coimmunoprecipitated FLAG-rSLM-1 was detected by the anti-rSLM-1 antiserum in Western blots. No coimmunoprecipitation was seen between EGFP and FLAG-rSLM-1. (D) The immunoprecipitated EGFP-fused proteins were detected by Western blot using an anti-GFP antibody to show their integrity. (E) Analysis of the antisera against rSLM-1 and rSLM-2. EGFP-rSLM-1, EGFP-rSLM-2, and EGFP-rSAM68 were over-expressed in HEK293 cells and lysates were analysed with affinity purified anti-rSLM-1 or anti-rSLM-2 antisera. Both antisera are specific for either rSLM-1 or rSLM-2 and do not recognize the related proteins rSAM68 or rSLM-2 and rSLM-1, respectively. Preabsorbtion of the antisera with the peptides used to generate them abolish the signal. (F) Detection of endogenous protein using the rSLM-1 and rSLM-2 antisera. Lysates from Neuro2A cells were analyzed by Western blot using the rSLM-1 and rSLM-2 antisera. Preabsorbtion of the antisera abolished the signal.

(Stoss et al., 2001). Since rSLM-1 and rSLM-2 share 66% sequence identity on the protein level, we assumed a similar role for rSLM-1. To investigate a possible role of rSLM-1 in the regulation of alternative splicing, we performed transient transfection assays with the CD44-v5 minigene (Stoss et al., 2001; Weg-Remers et al., 2001) that was previously shown to be regulated by rSLM-2 (Stoss et al., 2001). The minigenes contained the alternative spliced exon v5 flanked by two constitutive heterologous insulin exons under the control of a CMV promoter (Fig. 3A, left). A constant amount of the minigene was cotransfected with an increasing ratio of EGFP-rSLM-1 or EGFP-rSLM-2 to EGFP in HEK293 cells (Fig. 3B). Total RNA was isolated 18 h after transfection and RT-PCR was performed using minigene-specific primers. As shown in Fig. 3B, both rSLM-1 and rSLM-2 promote inclusion of the alternative CD44 exon v5. No statistical significant difference between the two factors could be observed. Like rSLM-2, rSLM-1 binds to a purine-rich RNA element (data not shown) that acts as a splicing enhancer in CD44 exon v5 (König et al., 1998; Stoss et al., 2001). We wanted to determine whether rSLM-1 acts through the same element and employed linker-scanning mutants that replace the enhancer (Fig. 3A, right). We found that replacing the purine-rich element ATGAAGAGGA by CGACGCGTCG in ls9 reduced the ability of rSLM-1 to promote exon v5 inclusion (Fig. 3C). Replacing the adjacent sequences in ls8 and ls10 had no statistical significant effects. These data show that similar to



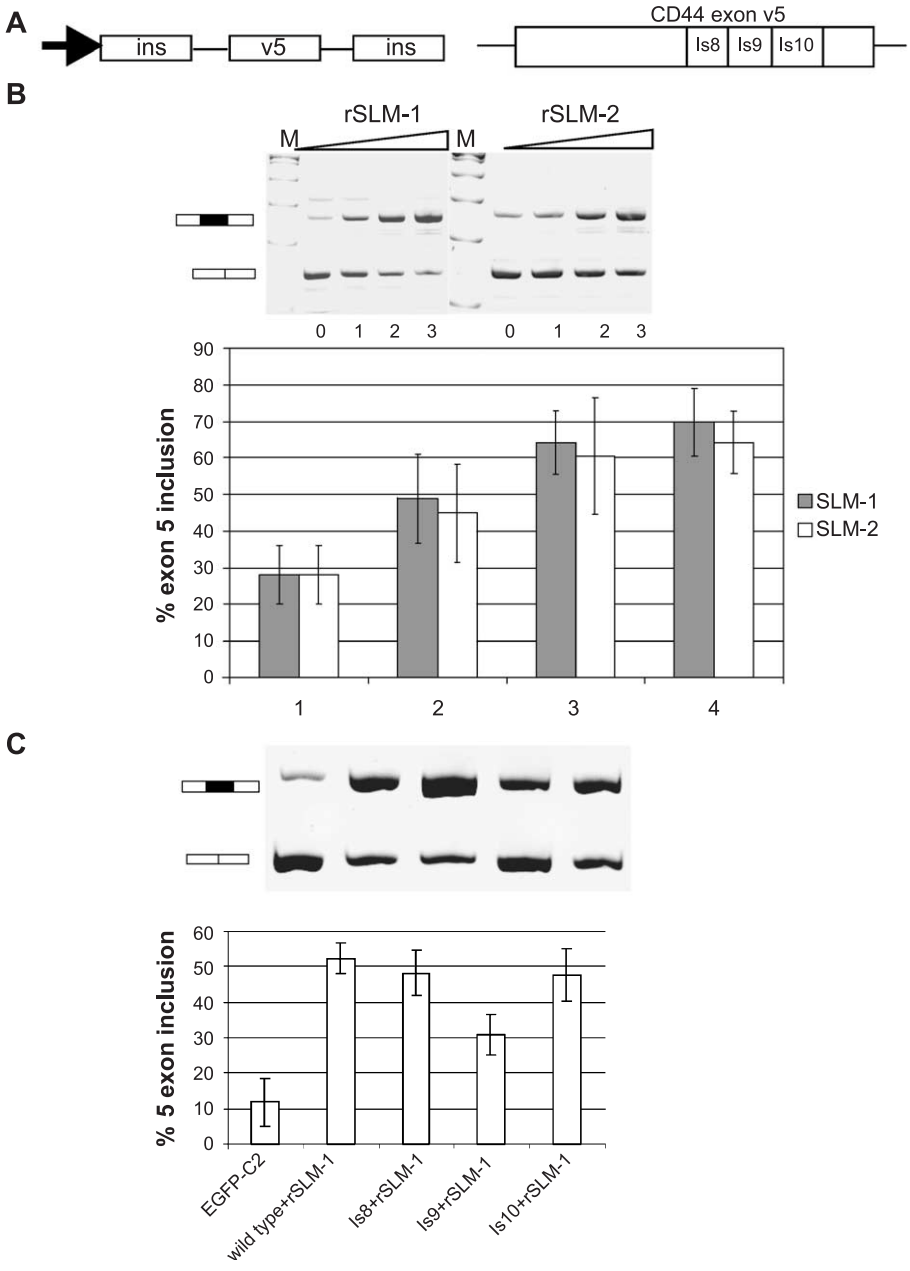


Fig. 3. rSLM-1 changes alternative splice site selection in vivo. (A) Structure of the minigene. The CD44v5 minigene containing the alternative-spliced exon v5 and flanking constitutive insulin exons is schematically indicated on the left. The arrow represents the CMV promoter. The location of the linker-scanning mutations (König et al., 1998) is schematically indicated on the right. (B) Increasing amounts of EGFP-rSLM-1 or EGFP-rSLM-2 were cotransfected with the CD44v5 minigene in HEK293 cells. The ethidium bromide-stained PCR products were quantified and result of four independent experiments is shown underneath. Dark bars: rSLM-1, lighter bars: rSLM-2. Error bars indicate the standard deviation. (C) The purine-rich element in exon v5 is necessary for rSLM-1 function. Two micrograms of EGFP-rSLM-1 were cotransfected with the wild type and linker scanning mutations indicated.

rSLM-2 (Stoss et al., 2001), rSLM-1 acts on a purine-rich element present in exon v5. Together with the binding data, this functional analysis shows that rSLM-1 and rSLM-2 have almost indistinguishable properties.

rSLM-1 and rSLM-2 show different tissue-specific expression

rSLM-1 and rSLM-2 share almost identical molecular properties, molecular binding partners and exert similar effects on splice site selection. To address the question why an organism expresses two highly related proteins, we compared the tissue distribution of rSLM-1 and rSLM-2 by Northern blot. The corresponding cDNAs lacking the first 311 nucleotides were used as hybridization probes. These cDNA fragments lack the most conserved part of the KH domain, which allows the discrimination of the related STAR proteins. We found that rSLM-1 is expressed only in brain and testis. In testis, two weak signals can be detected. One signal corresponds to a faster migrating mRNA, which could represent a

shorter rSLM-1 variant or a cross-hybridization to another mRNA. In addition, a very faint signal of the expected size is visible (Fig. 4A). In contrast, rSLM-2 is predominantly expressed in testis, brain, and heart and can be detected in all other tissues examined (Fig. 4B). In several systems, it was observed that no protein was generated from mRNAs, most likely due to translational control. We therefore investigated the protein expression by Western blot using lysates from different CNS areas and testis. As shown in Figs. 4D and E, rSLM-1 and rSLM-2 protein can be detected in all CNS areas, as well as in testis. However, in agreement with the mRNA expression, rSLM-1 is less abundant in testis than in brain. So far, our analysis did not allow for a direct comparison of the expression levels, as different probes had to be used. To obtain comparable data, we used DNA array analysis to determine the mRNA expression of SAM68, rSLM-1, and rSLM-2 in various tissues. As shown in Fig. 4F, SAM68 is the most abundant form in all tissues, except brain and testis. In all tissues, rSLM-1-mRNA is far less abundant than rSLM-2 or SAM68 mRNAs.

We conclude that from the three related proteins SAM68, rSLM-1, and rSLM-2, rSLM-1 shows the most restricted expression pattern and is the least abundant RNA, suggesting a specialized function predominantly in the brain.

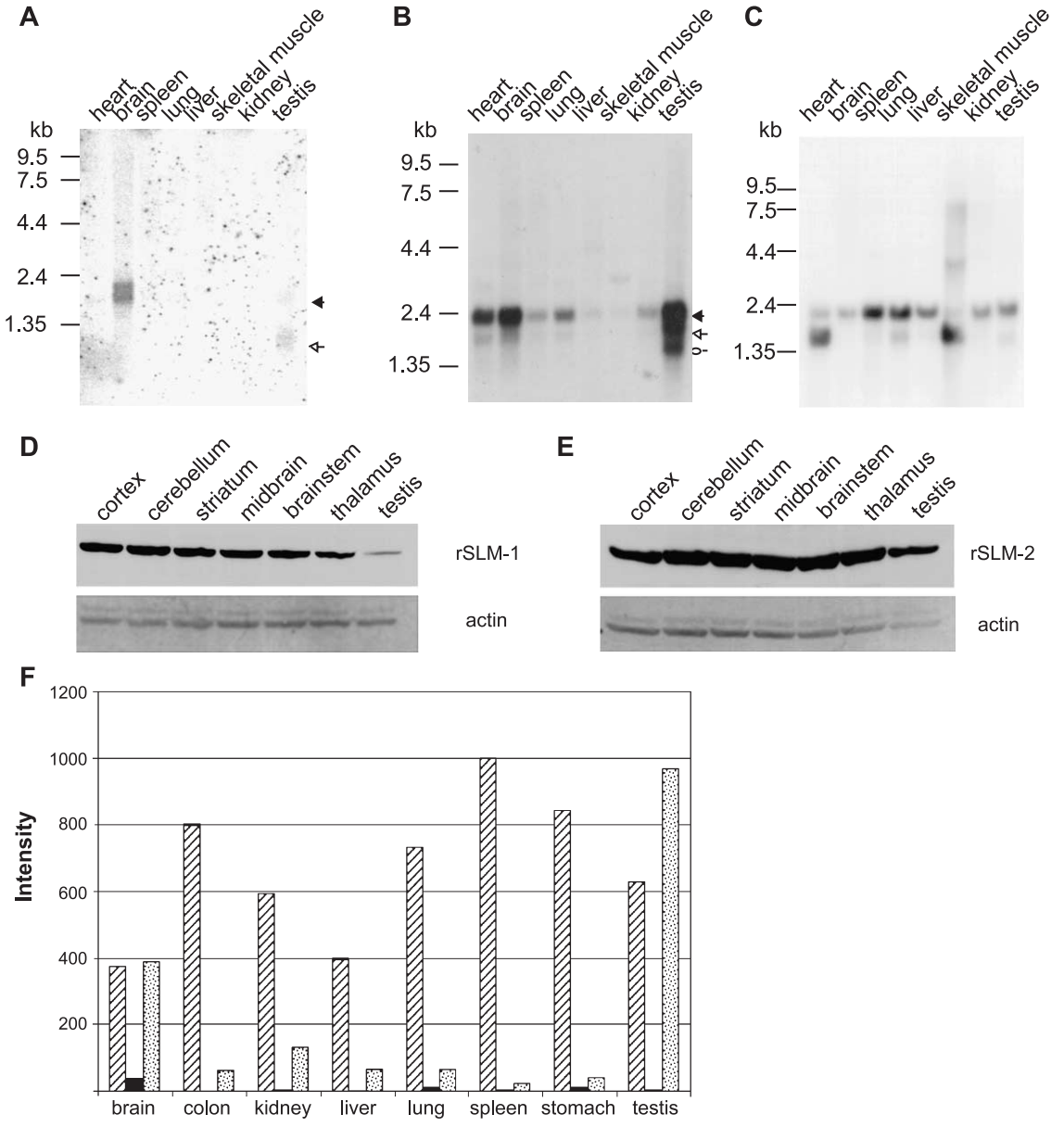


Fig. 4. Expression of rSLM-1 and rSLM-2. (A) A Northern blot with mRNA from different rat tissues was probed with rSLM-1, (B) the same blot was probed with rSLM-2, (C) the blot was probed with beta actin to demonstrate loading in each lane. (D) Western blot with lysates from different brain regions analyzed with rSLM-1 antiserum (top) and an antibody against actin (bottom). (E) Western blot with lysates from different brain regions analyzed with rSLM-2 antiserum (top) and an antibody against actin (bottom). (F) Array analysis of the expression of SAM68 (stripes), SLM-1 (black) and SLM-2 (dotted) with RNA from the tissues indicated.

rSLM-1 and rSLM-2 show non-overlapping neuronal expression in the brain

Northern blot analysis showed that both rSLM-1 and rSLM-2 are expressed in the brain. To investigate possible functional differences, we used immunocytochemistry to investigate their cellular expression patterns in the forebrain. Both proteins were expressed in the cortical layers 2–6. Staining of adjacent section indicated that the proteins were not expressed in the same cells. We did not observe costaining in other brain sections and in the peripheral nervous system (data not shown). However, in the hippocampus, we found a striking difference in the expression of rSLM-1 and rSLM-2 (Fig. 5). In the hippocampus, rSLM-1 is predominantly expressed in the dentate gyrus. However, only a few cells express rSLM-1 in the CA1 region and no rSLM-1-positive cells could be detected in the CA4 region (Figs. 5A, B). In contrast, rSLM-2 was highly expressed in the CA1 to CA4 regions (Figs. 5C, D), but almost no cells were positive in the dentate gyrus (Fig. 5D). To determine the identity of the rSLM-1- and rSLM-2-expressing cells, we examined the non-overlapping expression pattern of both proteins in the dentate gyrus and CA4 region in detail (Fig. 6). The higher magnification shows that most cells in the dentate gyrus express rSLM-1, whereas most cells in the CA4 region express rSLM-2. The few rSLM-1-positive cells in the CA4 region are rSLM-2-negative (Figs. 6A–F), which indicates a non-overlapping expression pattern. The morphology and location of the rSLM-1- and rSLM-2-positive cells suggest that they are neurons. We therefore performed double staining experiments with GFAP. As shown in Figs. 6G–N, rSLM-1 and rSLM-2 were absent from GFAP-positive cells. To further demonstrate that both proteins are expressed in neurons, we performed double staining with the neuronal marker NeuN and found that SLM-1-positive (Figs. 6O–Q) and SLM-2-positive (Figs. 6R–T) cells express NeuN. However, not all NeuN-positive cells express either SLM-1 or SLM-2, demonstrating a specific neuronal expression pattern. No specific signal was observed when preabsorbed antibodies were used. These experiments show that rSLM-1 and rSLM-2 have non-overlapping expression patterns in the brain and are expressed in neurons. Therefore, although rSLM-1 and rSLM-2 have almost identical biochemical properties (Figs. 2, 3), they are expressed in different cells. Due to this physical separation, they will not heteromultimerize in brain cells.

rSLM-1, but not rSLM-2 is phosphorylated by p59^{fyn}

Both rSLM-1 and rSLM-2 contain proline-rich regions and a tyrosine-rich carboxy terminus that could bind to SH3 and SH2 domains, respectively. Similar to Sam68, the proteins could therefore be substrates of tyrosine kinases. We tested whether the non-receptor tyrosine kinases c-src, p59^{fyn} and hFer phosphorylate rSLM-1, and rSLM-2 in vivo. EGFP-rSLM-1 and EGFP-rSLM-2 were cotransfected with expression constructs of these kinases and immunoprecipitated with an anti-GFP antibody. The immunoprecipitates were analyzed using an anti-phosphotyrosine antibody, PY20. Both proteins were strongly phosphorylated by c-src (Fig. 7A). In contrast, p59^{fyn} only phosphorylated rSLM-1, although equal amounts of rSLM-1 and rSLM-2 were present (Fig. 7A, GFP reblot). None of the proteins were phosphorylated by overexpressing hFer. All the kinases tested were active, as demonstrated by their ability to phosphorylate different proteins in cell lysates (Fig. 7A, right). In vivo, a phosphorylation of rSLM-1 by p59^{fyn} is only possible when both proteins are expressed in the same cell. It was shown by RNA in situ hybridization that p59^{fyn} is widely expressed in neurons and oligodendrocytes in the adult brain (Umemori et al., 1992), but no analyses of the protein expression have been reported. We therefore determined rSLM-1 and p59^{fyn} protein expression by immunohistochemistry. p59^{fyn} protein expression was detected in all neurons of the hippocampal formation. As

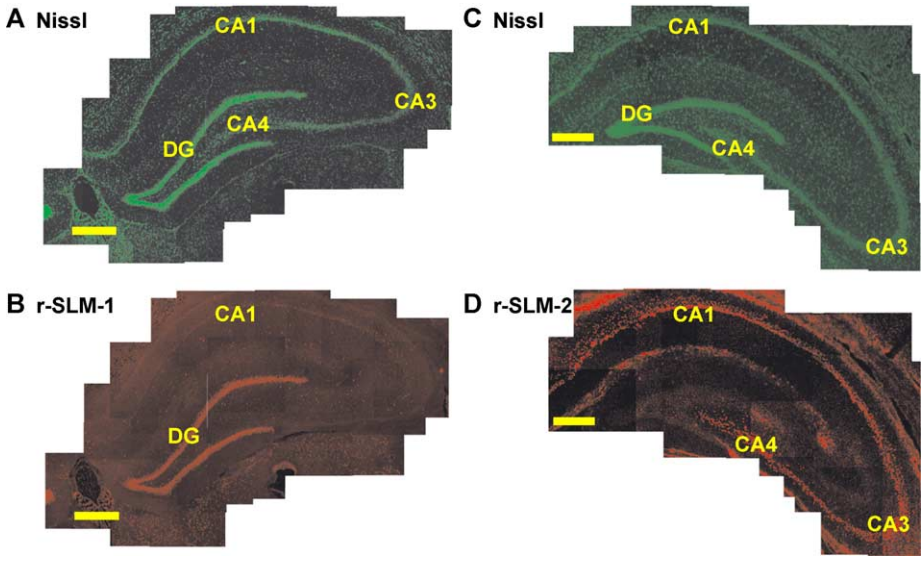


Fig. 5. Immunohistochemical analysis of rSLM-1 and rSLM-2 expression in the hippocampus. Ten-micron sections from adult rat brain hippocampus were analyzed with affinity purified rSLM-1 and rSLM-2 antisera. The CA1, CA3, and CA4 regions and the granule cells of the dentate gyrus (DG) are indicated. The scale bar represents 500 μm. (A, C) Neuro-trace green fluorescent Nissl staining of all nuclei. (B) Anti-rSLM-1 antiserum staining. (D) Anti-rSLM-2 antiserum staining.

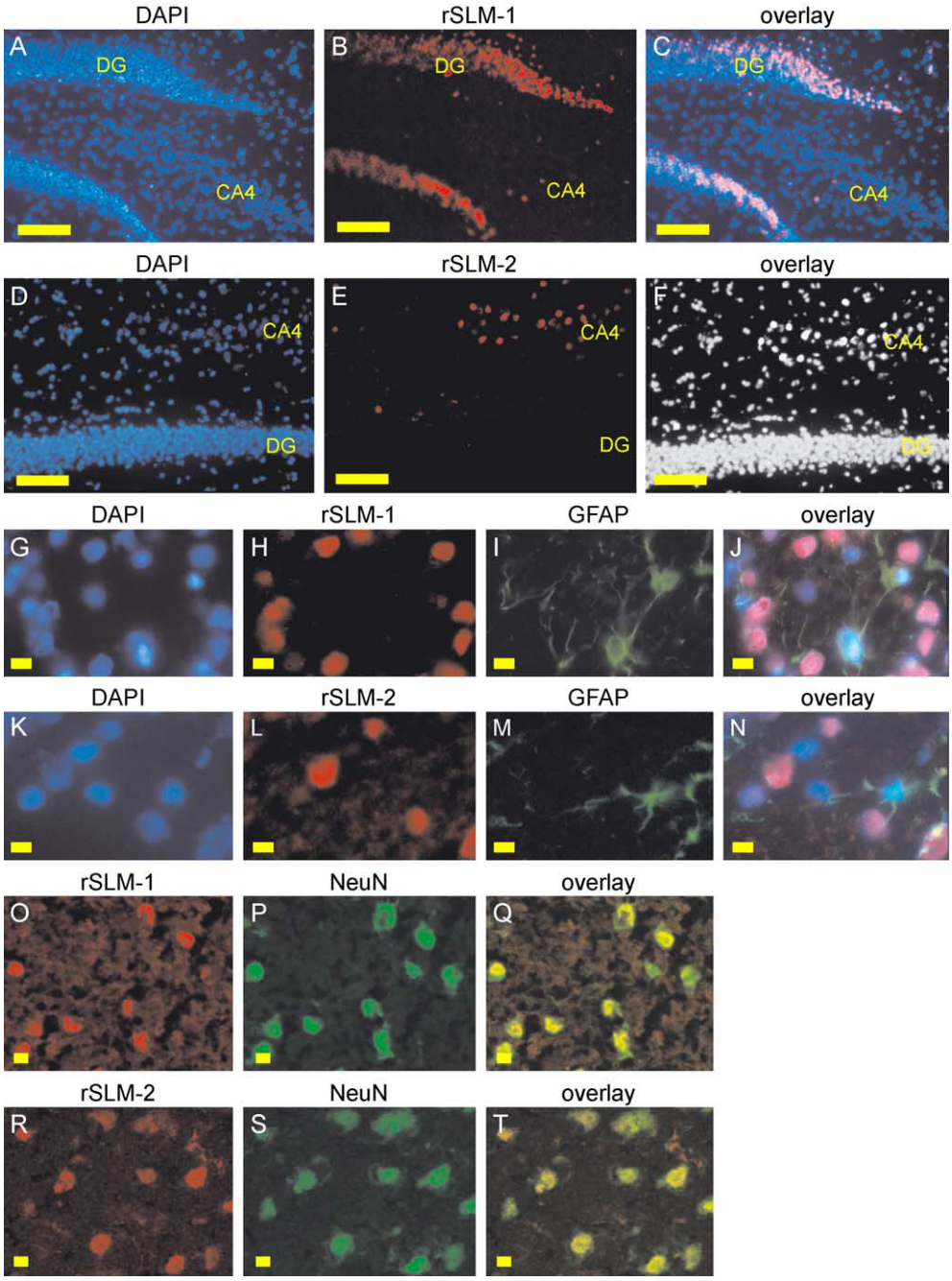


Fig. 6. Comparison of rSLM-1 and rSLM-2 expression in the CA4 region and in the dentate gyrus. Ten-micron sections from adult rat brain were stained with the anti-rSLM-1 or anti-rSLM-2 antisera. Nuclear staining by DAPI is shown in blue; the rSLM signals are shown in red; GFAP staining is shown in green. (A, D) DAPI staining of the hippocampal region. The CA3 and CA4 regions (CA3, CA4) and the granule cells of the dentate gyrus (DG) are indicated. The scale bar represents 100 μm. (B) rSLM-1 is present in the granule cells of the dentate gyrus (DG) but is absent from the CA4 region. Scale bar represents 100 μm. (E) rSLM-2 is present in the pyramidal cell nuclei of the CA4 region but is absent from the dentate gyrus. Scale bar is 100 μm. (C, F) Overlay of the corresponding DAPI and rSLM-1 or rSLM-2 stainings. (G–N) Triple staining of cells from the neocortex with DAPI (G, K), anti-rSLM-1 antibody (H) or rSLM-2 antibody (L) and anti-GFAP antibody (I, M). The corresponding overlays are shown in (J) and (N). rSLM-1 and rSLM-2 are not expressed in GFAP-positive cells. The scale bar represents 10 μm.

shown in Fig. 7B, within the dentate gyrus, rSLM-1 and $p59^{fyn}$ are expressed in the same cells. Our data thus show that rSLM-1, but not rSLM-2 is a substrate for $p59^{fyn}$ phosphorylation and that $p59^{fyn}$ and rSLM-1 are expressed in the same neuronal cells. Taken together, this suggests that $p59^{fyn}$ may regulate rSLM-1 function in the dentate gyrus.

rSLM-1 splicing properties are influenced by $p59^{fyn}$

The phosphorylation by $p59^{fyn}$ is a major difference between rSLM-1 and rSLM-2. We therefore asked whether $p59^{fyn}$-mediated phosphorylation would influence the ability of rSLM-1 to change splice site selection. As a more natural construct, we

employed the SMN2 minigene. In this minigene, the alternative exon 7 is flanked by its constitutive exons 6 and 8. Similar to CD44 exon v5, SMN2 exon 7 contains a purine-rich sequence element (Fig. 3), which acts as an exonic enhancer (Lorson and Androphy, 2000; Stoss et al., 2001). Since rSLM-1 is expressed in neurons, we performed the cotransfection assays in Neuro2A cells. We found that increasing the amount of either EGFP-rSLM-1 or EGFP-rSLM-2 expression constructs promotes skipping of the alternative exon 7 (Fig. 7C). The increase of the corresponding proteins was verified by Western blot analysis (Fig. 7C). The quantification of the results showed that there is almost no difference between the effect of rSLM-1 or rSLM-2. Given the $p59^{fyn}$-dependent phosphorylation of rSLM-1, we repeated this assay in the presence of a $p59^{fyn}$ expression construct. The presence of $p59^{fyn}$ reduced exon inclusion of SMN2 exon 7 from about 60% to 35% (Fig. 7D). Furthermore, $p59^{fyn}$ abolished the concentration-dependent ability of rSLM-1 to promote skipping of the alternative SMN2 exon. There was no statistical significant difference between the transfection results of various rSLM-1 concentrations when $p59^{fyn}$ was present. In contrast, the ability of rSLM-2 to promote skipping of the same exon was unchanged. Higher rSLM-2 concentrations increased exon skipping from 35% to 12%, a statistical significant change ($P = 0.001$, $t = 8.23$). In this assay, rSLM-2 is most likely not affected by $p59^{fyn}$ because it is not phosphorylated by $p59^{fyn}$ (Fig. 7A). Similar results were obtained when we used HEK293 cells (data not shown). Together, these experiments show that $p59^{fyn}$ mediated phosphorylation of SLM-1 can modulate its influence on splice site selection.

Discussion

rSLM-1 regulates splice site selection

The proper recognition and tissue-specific regulation of alternative exons is achieved by multiple, intrinsically weak interactions of proteins with sequences on the pre-mRNA (Maniatis and Reed, 2002). Since the 5′ and 3′ splice sites of naturally occurring mammalian exons often strongly deviate from the consensus (Stamm et al., 2000), additional elements are needed. These elements are present in the exon or its flanking introns and can act as either enhancers or silencers (Hastings and Krainer, 2001). The elements are recognized by RNA-binding proteins which results in the formation of a commitment complex that is recognized by the spliceosome (Reed, 1996). Therefore, a tissue-specific expression of a protein can result in a tissue-specific exon usage. For example, the female specific exon 4 of doublesex in *Drosophila* has a regulatory element, DSX, which binds to the SR-protein RBP1 and tra-2 only when the female specific tra protein is expressed (Lynch and Maniatis, 1996).

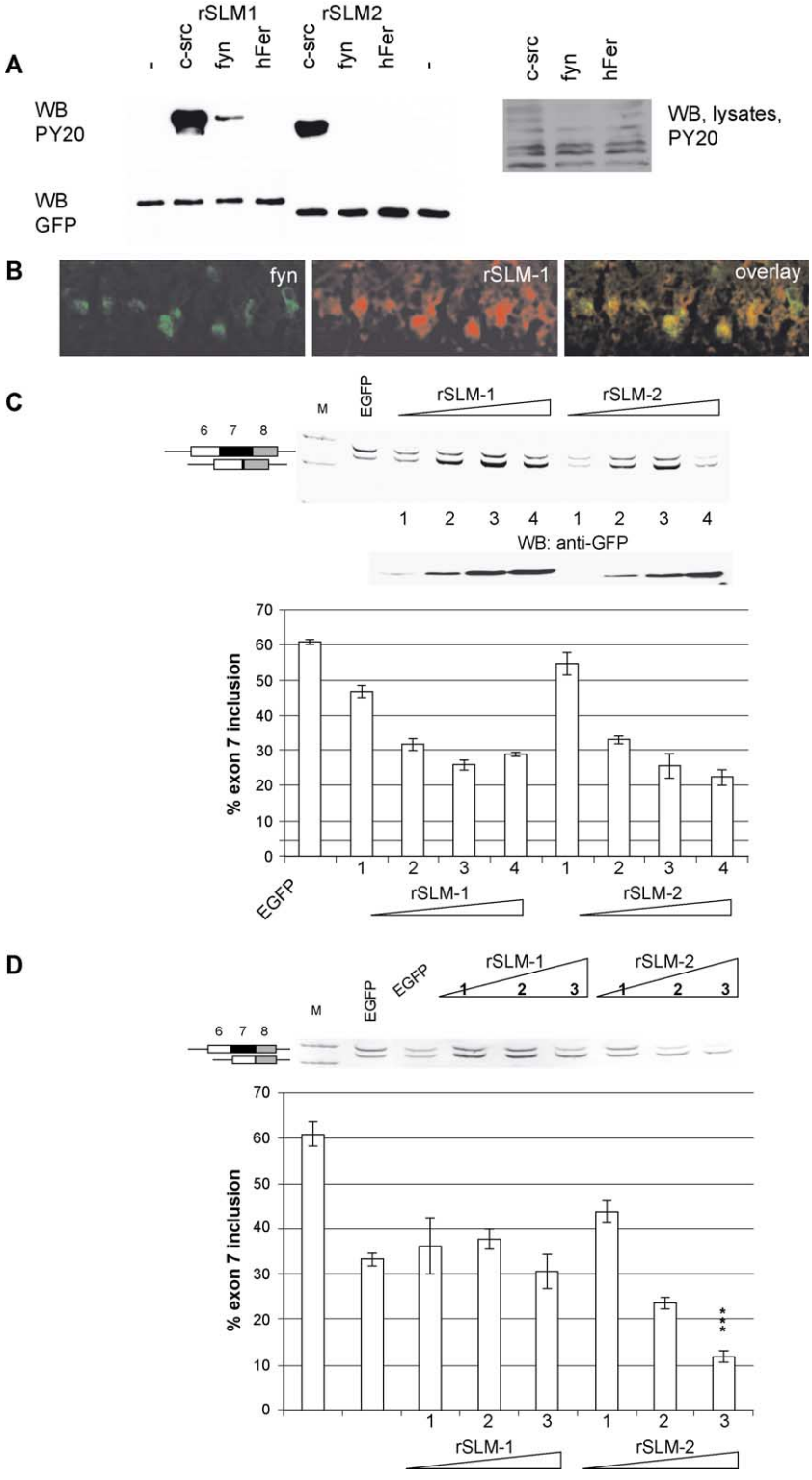
Here, we show that the highly related proteins rSLM-1 and rSLM-2 have similar sequences and almost identical biochemical properties. Similar to rSLM-2, rSLM-1 acts on purine-rich enhancer sequences present in the alternative exons of CD44 and SMN2, indicating that both proteins could regulate the same alternative exons. Both splicing factors bind to scaffold attachment factor B, SRp30c, YT521-B, hnRNP G, SLM-1, SLM-2, and SAM68. All these proteins bind to pre-mRNA: hnRNP G, SRp30c, and SAF-B bind RNA via their RNA recognition motif (Screaton et al., 1995; Soulard et al., 1993; Weighardt et al., 1999), the STAR proteins rSLM-1, rSLM-2, and SAM68 bind RNA via their KH domain and YT521-B contains a putative nucleic acid binding domain, YTH (Stoilov et al., 2002). It is therefore likely that in combination these proteins regulate alternative splicing by acting similar to the RBP1/tra2/tra and SF2/tra2/tra complexes (Lynch and Maniatis, 1996), hnRNP F,H/KSRP complexes (Markovtsov et al., 2000) and FBP/SAM68/PTB complexes (Grossman et al., 1998) that regulate alternative splicing of doublesex exon 4, the src N1 exon, and beta-tropomyosin exon 7, respectively. We sustained this hypothesis by overexpressing rSLM-1 and rSLM-2 with reporter genes and found that they can regulate splice site selection of the SMN2 pre-mRNA in a concentration-dependent manner. SMN2 exon 7 is regulated by SRp30c (Young et al., 2002) and hnRNPG (Hofmann and Wirth, 2002). This suggests that the observed binding properties of rSLM-1 and rSLM-2 are reflected in the formation of regulatory complexes in vivo. Exon regulation by rSLM-1 and rSLM-2 appears to be specific, as several other alternative exons, such as clathrin light chain B, exon EN (Stamm et al., 1999), or neurofilament tau exon 10 (Wang et al., 2004) are not affected (data not shown).

rSLM-1 is expressed only in neurons and testis

Since rSLM-1 and rSLM-2 have very similar properties, we asked why mammals express two highly related proteins. The first difference we observed was the expression pattern. In agreement with the situation in human (Di Fruscio et al., 1999; Sugimoto et al., 2001), rSLM-2 is expressed in several tissues. However, different from earlier work in mouse (Di Fruscio et al., 1999), we find rSLM-1 expression exclusively in rat brain and testis. Since the non-brain signal in mouse has a lower molecular weight (Di Fruscio et al., 1999), the additional signal could be due to cross hybridization or a different rSLM-1 isoform. We then examined the protein expression in

Fig. 7. The tyrosine phosphorylation of rSLM-1 affects splice site selection. (A) Tyrosine phosphorylation of rSLM-1 and rSLM-2 by non-receptor tyrosine kinases. Three micrograms of EGFP-rSLM-1 and EGFP-rSLM-2 were coexpressed with 1 μg c-src, $p59^{fyn}$, and hFer in HEK293 cells and immunoprecipitated with an anti-GFP antibody. The precipitates were analysed using the anti-phosphotyrosine antibody PY20 using Western-Blot (WB). −, no kinase expressed. Immunoprecipitates were reblotted with anti-GFP to show equal loading. Crude cellular lysates transfected with the kinases were analysed with PY20 and are shown on the right. (B) $p59^{fyn}$ and rSLM-1 are coexpressed in the dentate gyrus. The nuclear layer of the dentate gyrus was stained with anti-$p59^{fyn}$ (green) and rSLM-2 antiserum (red). The overlay shows the localization of both proteins in the same cells. (C) Influence of rSLM-1 and rSLM-2 on SMN2 splicing. Increasing amounts of EGFP-rSLM-1 or EGFP-rSLM-2 were cotransfected with the SMN2 minigene in Neuro2A cells. A representative ethidium bromide stained gel in shown on top. The amount of protein expressed from the EGFP-rSLM-1 and EGFP-rSLM-2 constructs was determined by Western blot using the anti EGFP antiserum and is shown underneath (WB: anti-GFP). The bar diagram shows the statistical evaluation of at least four independent experiments. Numbers indicate μg expression construct transfected. Cartoons on the left side of the ethidium bromide-stained gels indicate the structure of the amplified isoforms. The black box shows the alternative exon. (D) In vivo splicing assay in the presence of $p59^{fyn}$. The SMN2 minigene was cotransfected in Neuro2A cells with increasing amounts of rSLM-1 and rSLM-2 in the presence of a $p59^{fyn}$ expression construct. The diagram shows the result of four independent experiments, similar to the one in (C). Stars indicate a statistical significant difference between the SLM-2-transfected cells as determined by the Student's *t* test (95% confidence interval, $P < 0.001$, $t = 8.23$).

the brain in more detail and found that both rSLM-1 and rSLM-2 are expressed in neurons. Both proteins are expressed in the cortex, but most likely not in the same cells. Staining of adjacent sections revealed that the proteins are expressed in different neurons. In the hippocampus, rSLM-1 is typical for cells of the dentate gyrus, whereas rSLM-2 is expressed in the CA1, CA3, and CA4 region, where rSLM-1 is absent. Within the hippocampal formation, rSLM-1 is therefore a cellular marker for the dentate gyrus. Other highly related splicing regulatory proteins, such as NOVA-1 and NOVA-2 (Yang et al., 1998) show non-overlapping expression patterns, which could be a mechanism to establish cell type-specific splicing in the brain. We searched for posttranslational modifications of the STAR proteins and found that rSLM-1 is tyrosine phos-



phorylated by $p59^{fyn}$, whereas this tyrosine kinase had no detectable effect on rSLM-2. To test the physiological importance of the $p59^{fyn}$-mediated phosphorylation, we performed cotransfection assays in the presence of $p59^{fyn}$. Expression of $p59^{fyn}$ abolished the rSLM-1-mediated exon repression, but had no effect on the function of rSLM-2 under the same conditions. We observed this effect both in neuronal and fibroblast cell lines, which indicates that it is an intrinsic property of rSLM-1.

Alternative splicing can be influenced by tyrosine kinase mediated phosphorylation

Cells dynamically regulate their selection of alternative splice sites. For example, the alternative usage of numerous exons changes during brain development (Stamm et al., 1994). This regulatory plasticity is not confined to the development, as external cues can regulate alternative splicing in the adult brain. For example, neuronal activity evoked by pilocarpin (Daoud et al., 1999), kindling (Kamphuis et al., 1992), cocaine treatment (Berke et al., 2001), or pavlovian fear memory (Stork et al., 2001) change alternative splicing patterns. Although some cis-elements, for example, in the STREX exon have been determined, (Xie and Black, 2001), the molecular details how alternative splicing is linked to signal transduction are still not clear (Stamm, 2002).

Our results provide evidence that tyrosine phosphorylation of a splicing regulatory protein can change splice site selection. Phosphorylation of rSLM-1 abolished the normal 1.5- to 2-fold modulation of alternative exon usage. Studies on Ras-dependent threonine phosphorylation of SAM68 revealed effects of comparable magnitude (Matter et al., 2002). Furthermore, stimulation-dependent changes of alternative splicing in vivo have similar 1.5- to 4-fold effects (Abdennebi et al., 2002; Holdiman et al., 2002; Meshorer et al., 2002; Stamm, 2002). This indicates that the effects we see in the transfection assays are comparable to the in vivo situation. The exact mechanism by which tyrosine phosphorylation changes alternative splice site selection remains to be determined. We previously demonstrated that sequestration of splicing factors can change splice site selection (Stoilov et al., 2004). Our preliminary data indicate that the protein interactions of rSLM-1 are modulated by phosphorylation, suggesting a phosphorylation-dependent sequestration as a likely mechanism that remains to be tested.

Whereas threonine residues of STAR proteins can be phosphorylated by extracellular signal-regulated kinase shown to reside in the nucleus, it is not clear how the tyrosine-phosphorylation signal generated by membrane bound $p59^{fyn}$ reaches rSLM-1 in the nucleus. Possible mechanisms include shuttling of rSLM-1 between nucleus and cytosol in hippocampal neurons, although we did not observe shuttling in fibroblasts (data not shown); different nuclear kinases activated by $p59^{fyn}$ and direct nuclear translocation of plasma-membrane integral tyrosine kinases that has been observed in several cases (Lin et al., 2001; Marti and Wells, 2000; Ni et al., 2001; Offterdinger et al., 2002; Wells and Marti, 2002). In fact, after overexpression $p59^{fyn}$ can be detected in the nucleus and endogenous $p59^{fyn}$ was observed in the nuclei of T-lymphocytes (Ley et al., 1994; Shima et al., 2001).

Disregarding the exact signaling mechanism from $p59^{fyn}$ to the nucleus, our data show that rSLM-1 influences splice site selection depending on its phosphorylation status. This suggests that the physiological role of rSLM-1 may be to allow $p59^{fyn}$ to change splicing patterns in a subset of neurons. This role explains why in addition to rSLM-2, a highly related protein is expressed in the brain. We suggest that SLM-1 is part of a signal transduction pathway linking extracellular cues to pre-mRNA processing. Tyrosine phosphorylation-dependent alternative splicing provides a model for the observation that activation of receptor tyrosine kinases by growth factors can change alternative splicing (McKay et al., 1994; Scotet and Houssaint, 1998; Sell et al., 1994; Wang et al., 1991) and that a change in alternative splicing is often correlated with cancer caused by tyrosine kinase misregulation.

Experimental Methods

Two-hybrid screening

The yeast two-hybrid screens and interaction experiments were performed as described (Fields and Song, 1989; Hartmann et al., 1999). Using rSAF-B as a bait in pGBT9, 200,000 colonies of a rat brain embryonic day P5 library (Stratagene, pAdGal4-cDNA as prey) were screened. The DNA of 15 lacZ-positive clones, able to grow on selective medium containing 10 mM 3-aminotriazole, was sequenced as described (Hartmann et al., 1999).

In vitro protein interaction assay

The cDNA of potential interactors of rSLM-1 was cloned in pCR3.1 (Invitrogen) and used for an in vitro reticulocyte lysate transcription/translation (TNT, coupled reticulocyte lysate system, Promega) to obtain the corresponding ^{35}S-labeled proteins. For the binding experiments, 2 μl of the reactions were incubated with 1 μg of GST or GST-rSLM-1 coupled to glutathione-Sepharose 4B (Amersham Pharmacia Biotech) in the presence of 200 μl of HNTG buffer/0.1% Triton X-100 (Stoss et al., 1999a,b) for 2 h at 4°C. Washing and detection was as described (Stoss et al., 1999a,b).

Antiserum production and purification

The following peptides were used to immunize rabbits after coupling to KLH: for rSLM-1 VNEDAYDSYAPEEWTTCG and DQTYEAYDNSYVTPTQSVPECG; for rSLM-2 VVTGKSTLRTRGVTCG and PRARGVPPTGYRPCG. After 121 days, serum was purified by affinity chromatography, employing a mixture of recombinant GST-rSLM-1 or GST-rSLM-2, respectively, following the manufacturer's instructions (Pierce). Dilution for Western blot was 1:1000 and for immunohistochemistry 1:200. Preabsorbtion of 10 μl anti rSLM-1 antiserum or rSLM-2 antibody at a concentration of 0.07 mg/ml with 80 μg of rSLM-1 peptides or rSLM-2 peptides for 30 min at room temperature abolished the specific signal.

Immunoprecipitation, coimmunoprecipitation and Western blot were performed as described (Hartmann et al., 1999). The following antibodies were used: anti-rSLM-1 (1:1000); anti-SAF-B (1:1000); anti-YT521-B (1:5000) (Hartmann et al., 1999); anti-GFP (Boehringer 1:5000); C20 (Santa Cruz Biotechnology, 1:2000); anti phosphotyrosine, PY 20 (1:10,000), anti-fyn (Santa Cruz Biotechnology, 1:50 for IHC).

Immunohistochemistry

Adult rat brain cryostate sections (10 μm) were fixed for 30 min in 4% paraformaldehyde in PBS. Permeabilization and blocking was

for 2 h with 0.5% Triton X-100 and 3% NGS in PBS. Incubation with the anti-rSLM-1- or anti-rSLM-2 antiserum (1:200 in PBS, 0.3% NGS, 0.5% Triton X-100) was over night at 4°C. After washing three times for 10 min with PBS, the tissue was incubated with a 1:200 dilution of a Cy3-coupled goat anti-rabbit-IgG antibody (Dianova) for 2 h followed by a counterstaining for 10 min with NeuroTrace green fluorescent Nissl stain solution (Molecular Probes). After three wash steps with PBS for 20 min, the tissue was embedded with Gelmount (Biomeda). The sections were analyzed by confocal microscopy (Leica).

Purification of recombinant rSLM-1 protein

The rSLM-1 cDNA was cloned in pGex4T1 (Pharmacia) and transformed in *Escherichia coli* BL-21. Overproduction was in 600 ml LB containing 50 μg/ml ampicillin. Protein expression was induced by 1 mM IPTG (Sigma) at an optical density (OD_{600}) of 1.0. The bacteria were then incubated for 1 h at 30°C. The cell pellet was resuspended in buffer A (PBS, 2 mM EDTA, 1% Triton X-100, 10% glycerol, 1 mM DTT, 1 mM PMSF, 20 μg/ml aprotinin, 2 μg/ml leupeptin, and 2 μg/ml pepstatin). Cell lysis occurred by repeated rounds of freezing and thawing with subsequent sonification for 30 s (Branson sonifier W450). After centrifugation for 30 min at 14000 rpm, the supernatant was mixed top over top with glutathione sepharose (Amersham) for 2 h at 4°C. After washing, the sepharose five times with buffer A, the protein was eluted with buffer A containing 0.5 M glutathione, dialysed against 1× PBS overnight and concentrated using centricon concentrators (Amicon).

In vivo splicing assays

Cellular splicing assays were performed as described (Stoss et al., 1999a,b), employing the CD44v5 (König et al., 1998) and the SMN-2 (Lorson et al., 1999) minigenes. The SMN-2 minigene contains the alternative exon 7 flanked by the constitutive exons 6 and 8 and the original introns and the control of a CMV promoter. Transfection of the minigenes occurred in HEK293 cells. The resulting splicing pattern was quantified using the Herolab EASY system.

Northern blot

A rat tissue Northern Blot (Clontech) was first hybridized with a radioactively labeled rSLM-2 cDNA probe lacking the first 311 nucleotides according to the manufacturer's instructions. The probe was removed and reprobed with a radioactively labeled rSLM-1 cDNA probe lacking the first 311 nucleotides. Finally, a beta-actin probe was used to verify loading in each lane. The membrane was then analyzed using a phosphoimager.

Array assays

Fifty-microgram total RNA was purified using RNeasy Mini columns (Qiagen) as described by the manufacturer. Five micrograms of purified total RNA was used for synthesis of first and second strand cDNA. Double-stranded cDNA was then used to synthesize the biotinylated cRNA probe as described by Affymetrix. Fifteen micrograms of fragmented labeled cRNA was hybridized to the human genome U133A and U133B arrays and stained with streptavidin-phycoerythrin (SAPE). These arrays contain 11 oligonucleotides per gene that bind to the 3′ UTR. The rat and human sequences are sufficiently conserved to allow detection. Signal intensities were amplified using a biotinylated anti-streptavidin antibody and a second SAPE staining step. Data were analyzed using the Microarray Suite software (MAS5, Affymetrix).

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