Splicing Factor Arginine/Serine-rich 17A (SFRS17A) Is an A-kinase Anchoring Protein That Targets Protein Kinase A to Splicing Factor Compartments*

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Elisabeth Jarnasen†‡, Anne Jorunn Stokka†§, Anne-Katrine Kvissel†, Bjørn S. Skålhegg¶, Knut Martin Torgersen†**, John D. Scott***, Cathrine R. Carlson†§, and Kjetil Taskén†§

From the †Biotechnology Centre of Oslo, the §Centre for Molecular Medicine Norway, Nordic European Molecular Biology Laboratory Partnership, the ¶Department of Nutrition, and the **Department of Biochemistry, Institute of Basic Medical Sciences, University of Oslo, PB 1125 Blindern, N-0317 Oslo, Norway and the ***Howard Hughes Medical Institute, Department of Pharmacology, University of Washington School of Medicine, Seattle, Washington 98195

The holoenzymes exhibit distinct cAMP binding affinities and localization inside cells and are classified as type I or type II based on their R subunit composition (4, 5).

PKA is targeted to distinct subcellular loci through interaction with a family of protein kinase A anchoring proteins (AKAPs). AKAPs encompass more than 50 structurally diverse but functionally related proteins that organize and target supramolecular signaling complexes and scaffold signaling pathways. AKAPs serve to organize discrete spatiotemporal regulation of signaling events mediated by PKA (1, 6). Although archetypical AKAPs bind type II PKA, more recent work shows that AKAPs can be defined by their ability to bind type I PKA (RI-specific) (7–9), type II PKA (RII-specific), or both (dual-specific) (10–15). Most AKAPs bind PKA through an amphipathic helix consisting of 14–18 amino acids (16–18) that inserts into a hydrophobic groove formed by the R dimer as evident from resolution of NMR and crystal structures of the complex (19–23). A structure-based substitution approach allowed identification of determinants for RI and RII binding to AKAPs and development of specific high affinity binding sequences (22, 24–26). We recently showed that some dual-specific AKAPs contain an additional binding region, the RI specifier region (RISR), that enhances anchoring of type I PKA (27). This region is detected in a variety of AKAPs, like Ezrin, Merlin, PAP7, D-AKAP1 and D-AKAP2, and provides a mechanism for multisite binding to the RI subunit, thus enhancing the affinity and specificity of type I PKA binding to AKAPs (27).

Upon activation of the PKA holoenzyme, a part of the C subunit pool enters the nucleus, where it is involved in regulation of transcription and splicing (29). Introns are removed from pre-mRNAs by the spliceosome, a massive complex that...
consists of five small nuclear ribonucleoprotein particles, U1, U2, U4, U5 and U6, associated with a large number of proteins. The spliceosome assembles in a stepwise manner via multiple RNA-RNA, RNA-protein, and protein-protein interactions. Components of the splicing machinery are recruited to sites of transcription from splicing factor compartments (SFCs), which are dynamic structures located within the nucleoplasm. Many pre-mRNA splicing factors, including small nuclear ribonucleoproteins and SR proteins, such as ASF/SF2 and SC35, localize to SFCs (30–32). In addition, SFCs contain several kinases and phosphatases that can modify the phosphorylation status of components of the splicing machinery. It has been suggested that SFCs are storage/assembly/modification sites for the pool of factors available to the transcription and pre-mRNA processing machinery (33).

SR proteins regulate both alternative and constitutive splicing. In addition to the serine/arginine-rich domain, SR proteins also contain an RNA recognition motif (34, 35). Previous studies have suggested that SFRS17A is an alternative splicing factor and an SR-related splicing protein that interacts with the classical SR protein ASF/SF2 and the SR-related factor ZNF265 (36). SFRS17A is ubiquitously transcribed and gives rise to two alternatively spliced isoforms of 695 and 385 amino acids (37). Whereas the long form involved in splicing has almost 30% arginine and serine in the last 300 amino acids, typical for SR proteins (37), the short form is suggested to be a target for degradation by nonsense-mediated mRNA decay and has, as of yet, no function assigned (38). SFRS17A was originally reported as the pseudoautosomal or X inactivation escape gene 7 (XE7) and as B-lymphocyte antigen precursor.

Here, we demonstrate that SFRS17A is a dual-specific AKAP that binds type I and type II PKA with high affinity and colocalizes with the PKA C subunit in nuclear speckles. Furthermore, we demonstrate that SFRS17A significantly influences the splicing pattern of the E1A minigene, whereas SFRS17A mutated in the PKA binding domain does not.

**EXPERIMENTAL PROCEDURES**

**Cell Cultures**—Jurkat cells expressing the SV40 large T-antigen (Jurkat TAg) and U-2 OS cells were cultured in RPMI 1640 (Invitrogen) supplemented with 10% fetal bovine serum, 100 units/ml penicillin, 1 mM pyruvate, and 1 × nonessential amino acids (complete medium). HEK293 and HEK293T cells were grown in complete Dulbecco’s modified Eagle’s medium. All cells were cultured at 37 °C with 5% CO2.

**Constructs**—Full-length SFRS17A was inserted into pFLAG-CMV-5a and pEGFP-N3. A truncated SFRS17A (amino acids 353–533) was inserted into pGEX-5X1 to generate a glutathione S-transferase (GST)-fused construct. Substitutions L438P, L439P, K445P, and K446P were introduced in the R-binding domain of SFRS17A-FLAG to abolish binding (SFRS17A mut-FLAG) by mutagenesis (QuickChange, Stratagene) using two different primer sets: SFRS17A(L438P/L439P) sense (5’-tgcccgcctgtgccgctgccgccggctgccggctggc-3’) and antisense (5’-agcaggatatgccgctgccgctgccggctggc-3’) and SFRS17A(K445P/K446P) sense (5’-tgcacatctgtacctggcgctgccggctggc-3’) and antisense (5’-gttgtcgtgtctcggcgctgccggctgccgagagctc3’). All constructs were confirmed by sequencing.

**Antibodies**—Polyclonal antibodies to SFRS17A were generated by Covance by inoculating New Zealand White rabbits with a keyhole limpet hemocyanin-tagged peptide, CNREPSCGGKGATGDDGL, corresponding to amino acids 579–595 of SFRS17A (AAA61304), using their standard protocol including a total of five peptide injections over a period of 118 days. Antibodies were affinity-purified by immobilizing immobilizing peptide on Affi-Gel® 10 (Bio-Rad), followed by column chromatography and elution with IgG elution buffer (Pierce). Purified antibody fractions were immediately adjusted to physiological pH by the addition of 1 × phosphate buffer (pH 7.0) before the antibodies were extensively dialyzed against TBS buffer. For immunoblotting the following antibodies were used: monoclonal mouse anti-AKAP 149 (1:500), mouse anti-GFP (1:1000), mouse anti-Rlα (1:250), and mouse anti-RIIα (1:500) from BD Biosciences and polyclonal anti-FLAG M2 (1:1000) from Cell Signaling. Mouse monoclonal anti-PKAα catalytic subunit (1:500) was from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA), whereas mouse monoclonal anti-Tβ (1:1000) was from Novagen.

**Protein Expression and Purification**—Bovine Rlα and human RIIα proteins were expressed in Escherichia coli BL21 and E. coli Rosetta, respectively, using 0.1–1.0 mM isopropyl-β-D-thiogalactopyranoside induction at room temperature (4 h) and purified on Rp-8-AHA-cAMP-agarose beads (BioLog) as described previously (39). GST-SFRS17A-(353–533) was expressed in E. coli Rosetta cells, induced using 0.5 mM isopropyl-β-D-thiogalactopyranoside at room temperature (4 h) and purified on glutathione-Sepharose beads (Sigma). The purified recombinant R proteins were dialyzed extensively against 20 mM Mops (pH 7) and 150 mM NaCl, and SFRS17A fused to GST was dialyzed against 50 mM Tris-HCl (pH 8) and 150 mM NaCl. Protein concentrations were determined using the Bradford protein assay and SDS-PAGE (10% gels) using BSA as a standard.

**Peptide Synthesis**—Peptides used for Surface Plasmon Resonance studies (RISR, ESKRRQEEAEQRK; RISR(Q6P/R12P), ESKRQEEAEPRK) were synthesized on an Intavis MultiPep robot (Intavis Bioanalytical Instruments AG) and verified by high performance liquid chromatography. Concentrations of the peptides were determined by amino acid analysis using an amino acid analyzer from Applied Biosystems. Immunizing peptide used for antibody production (SFRS17A-(579–595), CNREPSCGGKGATGDDGL) and the negative control peptide used for characterization of the SFRS17A antibody (SFRS17A-(167–176), KESGSEKPSEDVLVK) were produced by Novagen.

**Autospot Peptide Array**—Peptide spots were synthesized with Fmoc (N-(9-fluorenyl)methoxycarbonyl)-protection chemistry on cellulose membranes using a Multipet automated peptide synthesizer (Intavis Bioanalytical Instruments AG) as described (40).

**Presence of SFRS17A in Cell Lines**—RNA was isolated from different cell lines using RNeasy (Qiagen). First strand cDNA was synthesized using the iScript cDNA kit (Bio-Rad). PCR was performed using the primers SFRS17A forward (5’-GAGC-CAAGGCTTGAGACTA) and SFRS17A reverse (5’-CAGACGGAGCTCTGAACCTC) and a program involving 95 °C
for 5 min, 30 cycles of 94 °C for 1 
min, 56 °C for 45 s, 72 °C for 45 s, 
and finally 72 °C for 10 min, result-
ing in a PCR product of 445 bp. PCR 
products were analyzed on a 2% 
agarose gel stained with ethidium 
bromide.

Cell Fractionation—Subcellular 
protein fractionation from cellular 
components in HEK293T cells was 
performed using the Qproteome 
Cell Compartment kit according 
to the manufacturer’s instructions 
(Qiagen).

Immunoprecipitation—HEK293T 
cells at 50–80% confluence were trans-
formed with 5–10 μg of plasmid DNA 
(SFRS17A-GFP, empty vector GFP, 
SFRS17A-FLAG, SFRS17A(L438P/
L439P/K445P/K446P)-FLAG, or 
empty vector FLAG) per 10-cm dish 
using Lipofectamine® (Invitrogen) 
or FuGENE® 6 (Roche Applied Sci-
cence). Cells were lysed 24 h after 
transfection in lysis buffer (50 mM 
Tris-HCl (pH 7.4), 400 mM NaCl, 1 
mm phenylmethylsulfonyl fluoride, 
1 mM Na3VO4, 0.5% Triton X-100) 
with protease inhibitors (Complete 
Mini EDTA-free tablets; Roche 
Applied Science). Immunoprecipi-
tations were carried out using anti-
bodies against PKA-RIIα and/or 
PKA-RIα (BD Transduction Labo-
ratories). Immunocomplexes were 
was three times in lysis buffer 
before being subjected to SDS-
PAGE followed by immunoblotting 
with the indicated antibodies.

R Overlay—R overlays were con-
ducted as described (16), using 32P-
labeled recombinant murine RIIα 
(41) or recombinant bovine RIIα 
(A98S), substituted to allow auto-
phosphorylation (42). Briefly de-
scribed, the membrane with im-
mobilized peptide or protein was 
blocked in Blotto (5% (w/v) nonfat 
dry milk and 0.1% BSA in TBS. Puri-
fied recombinant R (4 μg) was 
radiolabeled with purified C sub-
unit of PKA (0.02 μg/μl) and 
[γ-32P]ATP (1.4 μCi/μl) in 50 mM 
Mops (pH 6.8), 50 mM NaCl, 2 mM 
MgCl2 and 1 mM dithiothreitol and 
separated from free [32P]ATP by gel 
filtration (G-50 Sepharose). Specific 
activity was quantified by liquid
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scintillation counting (1600TR Tri-Carb, Packard Instrument Co.). All overlays were incubated overnight at room temperature using $1 \times 10^6$ cpm/ml TBS, 0.01% Tween 20 (TBS-T). For competition assays, soluble peptide was added to the radio-labeled RI$\alpha$ and incubated for 2 h before adding the membrane. The membrane was washed five times in TBS-T, and the signal was detected by autoradiography.

**Solid Phase Pull-down**—Lysate from 40 x 10$^6$ Jurkat TAg cells was incubated overnight at 4 °C with SFRS17A-RISR (RKELRERLLELILLSKPKD) or SFRS17A-RISR mut (RKELRERLLELILLSPPPDP) peptide synthesized in triplicates on cellulose membrane. The membranes were subsequently washed twice (20 min) in lysis buffer (50 mM Hepes(pH 7.4), 10 mM NaPP$_p$, 0.1% Triton X-100, 50 mM NaF, 100 mM NaCl, 5 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 1 mM Na$_3$VO$_4$) with protease inhibitors (Complete Mini EDTA-free tablets, Roche Applied Science) and twice in high salt lysis buffer (1M NaCl, 0.5% deoxycholate, 0.1% SDS) and incubated overnight at 4 °C with Rp-8-AHA-cAMP-agarose bead slurry in the presence or absence of 75 mM cAMP. Beads were subsequently washed twice (20 min) in lysis buffer (1 M NaCl). Bound PKA was eluted by boiling in SDS-PAGE sample buffer and analyzed by Western blotting with the indicated antibodies.

**cAMP Affinity Chromatography**—Transfected HEK293T cells (10-cm dish) were lysed in radioimmune precipitation buffer (50 mM Tris-HCl (pH 7.8), 500 mM NaCl, 1% Nonidet P-40, 0.5% deoxycholate, 0.1% SDS) and incubated overnight at 4 °C with Rp-8-AHA-cAMP-agarose bead slurry in the presence or absence of 75 mM cAMP. Beads were subsequently washed twice (20 min) in lysis buffer (50 mM Hepes(pH 7.4), 1.5 mM MgCl$_2$, 10 mM KCl, 0.5 mM NaCl, 0.1% Nonidet P-40, 1 mM dithiothreitol) with protease inhibitors (Complete Mini EDTA-free tablets, Roche Applied Science) and twice in high salt lysis buffer (10 mM Hepes, pH 7.4, 1.5 mM MgCl$_2$, 10 mM KCl, 0.5% Triton X-100, 50 mM NaF, 100 mM NaCl, 5 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 1 mM Na$_3$VO$_4$) for 15 min. The beads were subsequently washed five times in lysis buffer (50 mM Hepes (pH 7.4), 10 mM KCl, 0.5M NaCl, 0.1% Nonidet P-40, 1 mM dithiothreitol) with protease inhibitors. Bound RI$\alpha$ was eluted from the beads with 75 mM cAMP, pH 7.0, for 1 h at room temperature before they were subjected to SDS-PAGE and immunoblotting with indicated antibodies.

**Surface Plasmon Resonance**—The binding studies between PKA-R and SFRS17A were performed on a Biacore T100 instrument (Biacore Life Sciences/GE Healthcare Europe). CM5 chips (Biacore), coated with 8-AHA-cAMP (BioLog), were used to capture cAMP-free bovine RI$\alpha$, and human RI$\alpha$ subunits at a flow rate of 5 $\mu$l/min (surface immobilization level of 100–230 response units for each subunit) as described previously (39) in running buffers at pH 7.4 (for RI, 10 mM Hepes, 150 mM NaCl, 50 $\mu$M EDTA, 0.5 $\mu$M ATP, 10 mM MgCl$_2$, and 0.1% surfactant P20; for RII, 10 mM Hepes, 150 mM NaCl, 50 $\mu$M EDTA, and 0.1% surfactant P20). All subsequent interaction studies were performed in running buffers at pH 8 (for RI, 50 mM Tris, 150 mM NaCl, 50 $\mu$M EDTA, 0.5 $\mu$M ATP, 10 mM MgCl$_2$, and 0.1% surfactant P20; for RI, 50 mM Tris, 150 mM NaCl, 50 $\mu$M EDTA, and 0.1% surfactant P20) at 25 °C.

The fusion protein GST-SFRS17A-(353–533) was injected at a flow rate of 30 $\mu$l/min in a series of dilutions (from 62.5 to 2.0 nM; for 90 s) to determine the affinity of the interaction. After injection, the dissociation phase was monitored for 300 s. Nonspecific binding was subtracted using blank runs performed on a surface immobilized with 8-AHA-cAMP with no R subunit captured. Competition experiments were performed on immobilized bovine PKA-RI$\alpha$ or human PKA-RII$\alpha$ subunits. 50 nM GST-SFRS17A-(353–533) was injected in the presence or absence of 10 $\mu$M RISR or RISR(Q6P/R12P) at a flow rate of 30 $\mu$l/min for 90 s, and the binding level to the R subunit was analyzed. RISR peptide was also injected in the absence of AKAP. Kinetic analysis was performed using the Biacore T100 evaluation software.

**EIA Splicing Assay**—EIA splicing assays were performed as described (29). Briefly described, U-2OS cells grown in 6-well dishes were co-transfected with 0.5 $\mu$g of the splicing reporter minigene EIA and 1.5 $\mu$g of either Ca$_2$, SFRS17A, SFRS17A(L438P/L439P/K445P/K445P), ASF/SF2, or empty pFLAG-CMV-5a plasmid using FuGENE® 6 (Roche Applied Science). Twenty hours after transfection, RNA was isolated using RNasy (Qiagen). First strand cDNA was synthesized using the iScript cDNA kit (Bio-Rad). PCR was performed using primers EIA forward (5’-GTTTTTCCTCTCGAGGCCGCTC-GCA) and EIA reverse (5’-CTCAGGCTCAGGTTCAGACAC-GAG) and a program involving 95 °C for 5 min, 25 cycles of 94 °C for 30 s, 62 °C for 20 s, 72 °C for 40 s, and finally 72 °C for 10 min. PCR products were separated on a 1.5% agarose gel stained with Gelstar® (Cambrex). Protein extracts were prepared from the same samples to study expression levels of transfected plasmids.

**Immunofluorescence Analysis**—For immunofluorescence analysis, HEK293T cells were grown on coverslips coated with collagen and fibronectin (both from Sigma) for 48 h. At room temperature, cells were fixed with 3% paraformaldehyde in phosphate-buffered saline (PBS) for 15 min, permeabilized with 0.1% Nonidet P-40, PBS for 5 min, and then blocked for 30 min with 2% BSA, 0.01% Tween 20, PBS (PBST-BSA). The primary antibodies anti-rabbit SFRS17A (1:100; custom made), anti-mouse SC35 (1:100; Sigma), or anti-mouse PKA catalytic subunit (1:100; Santa Cruz Biotechnology, Inc.) in PBST-BSA were added for 30 min. Cells were then incubated with fluorochrome-conjugated secondary antibodies (Alexa Fluor 488 goat anti-mouse IgG, Alexa Fluor 488 goat anti-rabbit IgG, Alexa Fluor 546 goat anti-rabbit IgG, and Alexa Fluor 546 goat anti-mouse IgG (1:500); Molecular Probes) in PBST-BSA for 30 min before being mounted with glass coverslips using fluorescent mounting medium (DakoCytomation). Confocal microscopy was performed with a Zeiss LSM 510 META confocal microscope with a Plan-Apochromat 63 x 1.4 oil differential interference contrast objective lens, using laser excitation at 488 and 546 nm. Pictures were obtained using sequential scanning, and
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A

RI

Response Units

-10 0 10 20 30 40 50 60 70

Time (s)

-50 0 50 100 150 200 250 300

0 nM 2.0 nM 3.9 nM 7.8 nM 15.6 nM 31.3 nM 62.5 nM

B

RI

Response Units

-10 0 10 20 30 40 50 60 70

[5FRS17A] (nM)

0 20 40 60 80 100 120 140

KD (nM) = 16 ± 0.5

C

RII

Response Units

-10 0 10 20 30 40 50

Time (s)

-50 0 50 100 150 200 250 300

0 nM 2.0 nM 3.9 nM 7.8 nM 15.6 nM 31.3 nM 62.5 nM

D

RII

Response Units

-10 0 10 20 30 40 50

[5FRS17A] (nM)

0 20 40 60 80 100 120 140

KD (nM) = 21 ± 2.4

E

RI

Response Units

-10 0 10 20 30 40 50 60

Time (s)

-50 0 50 100 150 200 250 300

GST-SFRS1?A

GST-SFRS1?A + RISR

GST-SFRS1?A + RISR-P

RISR

F

RII

Response Units

-10 0 10 20 30 40 50

Time (s)

-50 0 50 100 150 200 250 300

GST-SFRS1?A

GST-SFRS1?A + RISR

GST-SFRS1?A + RISR-P

RISR

G

RISR

Response Units

-10 0 10 15 20

Time (s)

-50 0 50 100 150 200

Ri

RII
the exposure settings and gain of laser were kept the same for each condition.

Statistical Analysis—Data are presented as mean ± S.E. Paired statistical analyses were performed using Student’s t test. The two-sided p values were considered statistically significant at p < 0.05.

RESULTS

Identification of SFRS17A as an AKAP—We previously reported that dual-specificity AKAPs contain an additional binding region, RISR, increasing the affinity and specificity of type I PKA binding (27). A bioinformatics search in the International Protein Index data base, using a RISR consensus sequence (LX_3E_3X_3E) as probe, identified SFRS17A as one of nine proteins with a putative RISR (supplemental Table 1). We explored the possibility that SFRS17A could be a novel AKAP. To analyze SFRS17A binding to PKA, a family of 339 overlapping 20-mer peptides (offset by two residues) were synthesized on peptide array and subjected to overlay with radiolabeled RI and RII. RI bound three different regions in SFRS17A: residues 83–112 (VENKSLVKSFLACLGDGTIKLSGFSDILKV, R-binding region 1), residues 147–174 (DTIHLEGIPCKWALKESG-SEKPSDEVVL), and residues 425–454 (GLQRKERELRERLL-SILLSKPKDDSTHD, R-binding region 2) (Fig. 1A). The region covering residues 147–174, which did not contain any RISR or amphipathic helix structure but rather two helix-breaking prolines, did not appear to bind in subsequent analyses and was consequently excluded as a putative RI/RII binding site. The most N-terminal region (R-binding region 1) bound RII as well as RI (Fig. 1B) and, by modeling in α-helical configuration, appeared to contain an amphipathic helix. However, no RISR sequence was identified in this region. In contrast, the most C-terminal region (R-binding region 2) contained a putative RISR encompassing amino acids 426–437 (GLQRKERELRERLL), to which little RII binding was observed. Furthermore, R-binding region 2 was, by more detailed mapping using a combination of overlapping peptides offset by one residue and peptides with N- and C-terminal truncations, found to contain a partially overlapping sequence in residues 433–446 that could also be modeled as an amphipathic helix (supplemental Fig. S1, A–C).

R-binding region 2 in SFRS17A with the RISR sequence was next utilized in a solid phase pull-down assay, where immobilized 20-mer SFRS17A-RISR or negative control peptide, SFRS17A-RII mut, synthesized on membranes was incubated in a Jurkat TAg cell lysate. Substitutions in the control peptide were made based on the results obtained from a two-dimensional peptide array performed with the SFRS17A-RISR sequence (supplemental Fig. S2A). Bound proteins were eluted with SDS loading buffer, and the presence of PKA subunits was assessed by immunoblotting using specific antibodies. As shown in Fig. 1C, both PKA-RIα and PKA C subunits but not PKA-RIIα were pulled down by the SFRS17A-RISR peptide. Interestingly, PKA-R1o and PKA C also bound SFRS17A-RII in the reverse orientation. Having established that SFRS17A binds PKA in vitro, the SFRS17A-PKA interaction was then studied in situ with coprecipitation experiments. Full-length FLAG-tagged SFRS17A containing R binding regions 1 and 2 was expressed in HEK293T cells and cell lysate subjected to affinity chromatography on immobilized bovine Pre-mRNA Splicing

SFRS17A-RISR in the reverse orientation. Having established that SFRS17A binds PKA in vitro, the SFRS17A-PKA interaction was then studied in situ with coprecipitation experiments. Full-length FLAG-tagged SFRS17A containing R binding regions 1 and 2 was expressed in HEK293T cells and cell lysate subjected to affinity chromatography on immobilized bovine Pre-mRNA Splicing

FIGURE 2. Analysis of the kinetics of the SFRS17A-PKA interaction. A and C, SPR studies of the GST-SFRS17A-(353–533) binding to immobilized bovine PKA-RI (A) or human PKA-RII (C) on a sensor chip coated with 8-AHA-cAMP. GST-SFRS17A-(353–533) at the concentrations indicated was injected for 90 s, and the dissociation phase was monitored for 300 s. The dissociation graphs are representative of four independent experiments performed on different sensor surfaces. B and D, steady state binding of the increasing GST-SFRS17A-(353–533) concentrations obtained from A or C, respectively. The affinity constant (K_d) was derived, assuming a 1:1 Langmuir binding model using a global fit analysis algorithm provided by the BIAcore T100 evaluation software. E–G, qualitative surface competition experiments with the RISR peptide. 50 nM GST-SFRS17A-(353–533) was injected on a chip with captured bovine PKA-RI (E) or human PKA-RII (F) in the presence or absence of 10 μM RISR or RISR(Q6P/R12P) peptide. The RISR peptide was also injected without GST-SFRS17A present (G). The graphs show one representative experiment of three (or four) independent experiments performed on different chips.
Expression and Subcellular Localization of SFRS17A—Examination of SFRS17A expression by reverse transcription-PCR demonstrated that the SFRS17A mRNA was present in all cell lines tested (Fig. 3A). For immunolocalization studies of the endogenous SFRS17A protein, antibodies were generated by peptide immunization of rabbits. The specificity of the antibodies was first evaluated by immunoblotting against different amounts of spotted immunizing peptide. The crude SFRS17A antibodies showed high specificity to the peptide antigen (Fig. 3B, top) without any reactivity toward a negative control peptide derived from another region of SFRS17A (bottom). The specificity of the purified anti-SFRS17A was further evaluated by immunoblotting of cell lysates from HEK293T cells transfected with FLAG- or GFP-tagged SFRS17A (Fig. 3C). The polyclonal SFRS17A antibodies detected proteins with molecular masses corresponding to the predicted masses for the FLAG- and GFP-tagged SFRS17A, respectively.

Next, HEK293T cells were fractionated into nuclei, cytoplasm, membrane, and cytoskeletal fractions and examined for the presence of endogenous SFRS17A by immunoblotting. Endogenous SFRS17A was only observed in the nuclear fraction (Fig. 3D). The subcellular distribution of endogenous SFRS17A in HEK293T cells was further examined by immunofluorescence, which demonstrated specific staining of spots in the nucleus (Fig. 3E, top), consistent with previous observations (36). The specificity of the staining was confirmed by incubation with the immunizing peptide, which resulted in loss of staining (Fig. 3E, bottom).

SFRS17A Colocalizes with the PKA C Subunit and SC35 in HEK293T Cells—A subpopulation of the PKA C subunit has been shown to be localized to SFCs in the nucleus, where it colocalizes with the splicing factor SC35 (29). Furthermore, SFRS17A and SC35 colocalize in well defined spots (Fig. 4, A–D). With the use of our newly developed anti-SFRS17A antibodies combined with anti-C antibodies, we found both SFRS17A and the PKA C subunit to be colocalized and present in SFCs of HEK293T cells (Fig. 4, E–H). Overexpressed SFRS17A demonstrated a similar localization pattern (data not shown).
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Expression of SFRS17A with an Intact PKA Binding Domain Facilitates Splicing of the E1A Minigene—Introduction of four prolines in the R-binding region 2 of SFRS17A ([L438P/L439P/K445P/K446P]) abolished binding to PKA RI in a co-immunoprecipitation experiment (Fig. 5A). This mutant was further used as a negative control in an E1A pre-mRNA splicing experiment. The presence of three alternative 5’ splice sites in the E1A pre-mRNA results in three major mRNAs (13, 12, and 9 S) (43). In addition, two minor mRNAs (11 and 10 S) are created by usage of an additional internal 3’ splice site acceptor (44) (schematically illustrated in Fig. 5B). In U-2OS cells transfected with E1A and empty FLAG-vector, we detected 13 and 12 S mRNAs as the major mRNA products (Fig. 5D, lane 1), indicating that mRNAs generated by proximal splice site selection are mainly detected in U-2OS cells. Overexpression of T7-ASF/SF2 as a control resulted in elevated levels of the 13 S proximal splice product as expected, with the other mRNA products being essentially absent (Fig. 5D, lane 5) (45). The splicing analysis indicated that overexpression of SFRS17A-FLAG gives activation of the distal 9 S splice site, resulting in increased levels of 9 S mRNA products compared with the vector control (Fig. 5, D and E, p < 0.05) and decreased levels of the 13 S mRNA product (p < 0.05). This indicates that SFRS17A modulates the 5’ splice site selection from proximal to distal sites. Similarly, a shift to distal 5’ splice site selection was also observed in U-2OS cells transfected with PKA-C, as previously reported (29) (Fig. 5, D and E, p < 0.05). In contrast to the significant effect of SFRS17A wild type overexpression on the E1A splicing pattern, overexpression of SFRS17A mut only resulted in decreased levels of 13 S and increased levels of 12 S (Fig. 5, D and E, p < 0.05). Comparison of the SFRS17A wild type and mutant showed that the decrease of 13 S and increase of 12 and 10 S mRNA products were significant (Fig. 5E, p < 0.05). The requirement for the presence of the type I PKA binding site in SFRS17A for the regulatory effect on splicing indicates the involvement of type I PKA in the regulation of pre-mRNA splicing and that anchoring of type I PKA to the SFCs via SFRS17A may increase the efficacy of this process.

DISCUSSION

In this report, we demonstrate that the SFRS17A protein, encoded by the pseudoautosomal gene XE7, is a dual-specific AKAP. By peptide array and R overlay, SPR kinetic analysis, immunoprecipitations, solid phase pull-down, and cAMP affinity chromatography, we show that SFRS17A binds both type I and type II PKA. Furthermore, we demonstrate by immunofluorescence analysis that SFRS17A colocalizes with the C subunit of PKA and the splicing factor SC35 in nuclear speckles. Using the E1A minigene in a pre-mRNA splicing experiment, we also show that expression of wild type SFRS17A, but not SFRS17A substituted to abolish PKA binding, confers PKA-mediated regulation of pre-mRNA splicing.

Our recent discovery of the RISR, an additional PKA binding region in dual-specificity AKAPs, provided new means to classify this group of AKAPs (27). Furthermore, the RISR provided us with a new tool to explore existing data bases for novel, potential AKAPs by using the RISR-generated sequence as bait in a bioinformatic search. A short list of potential, novel AKAPs was unraveled from the search, among them the splicing factor SFRS17A.

Conventional AKAPs are classified by their ability to co-purify with the PKA holoenzyme from tissues or cell lysates. They typically contain a stretch of 14–18 amino acids forming an amphipathic helix that binds to the R subunit of PKA (16–18). SFRS17A co-purified with the PKA holoenzyme, as evident from immunoprecipitations, cAMP affinity chromatography, and solid phase pull-down and thus meets the criteria for being an AKAP. When performing a peptide array scan through the entire SFRS17A protein sequence, we observed three stretches of spots binding PKA. Following secondary structure prediction, the most N-terminal of the sequences turned out to be a typical amphipathic helix binding both type I and type II PKA, whereas the most C-terminal type I PKA binding sequence was the RISR-like sequence in SFRS17A, which also contained an amphipathic helix.

Alignment of SFRS17A with different AKAPs showed that the RISR of SFRS17A aligned with the PKA binding region of pericentrin both in the forward and the reverse direction (supplemental Fig. S2B). Although the 100-amino acid-long PKA binding region in pericentrin is shown only to bind type II PKA through a motif different from the amphipathic helix, it is interesting to speculate whether this long binding region also encompasses a repeated and inverted RISR, with a stretch of amino acids in between that confers RI specificity, possibly by binding to the hydrophobic groove in the D/D domain. The structure of the D/D domain of PKA RI consists of a symmetrically organized X-type antiparallel dimer forming the AKAP...
Due to the symmetry axis, the AKAP can bind to both R subunits in the hydrophobic groove of the R dimer in either orientation. This may provide the basis for why the SFRS17A RISR binds PKA when spotted in both directions (Fig. 1C) because it can contact either of the two R protomers. Furthermore, this symmetry could be why the RISR aligns with the PKA binding region of pericentrin in both forward and reverse sequence. Real time kinetics studies (SPR) of the SFRS17A-PKA interaction revealed that SFRS17A binds type I and type II PKA with similar affinities in the low nanomolar range. This is in contrast to most AKAPs studied so far because they have been shown to bind RII with higher affinity than RI due to the more dynamic state of the type I PKA complex, generally reflected in the dissociation rate, which is ~100-fold higher for RII than the RIa subunit (16, 24, 39). This formed the basis for our opinion that SFRS17A is a true dual-specific AKAP, binding equally well to both type I and type II PKA. A plausible explanation for this observation could be that the RISR contributes to the higher affinity for RI by providing multisite contact with the RI subunit that may stabilize the anchored type I PKA complex and thus compensates for the relatively lower affinity and faster off-rate of RI compared with RII binding to the amphipathic helix. We have previously shown that the RISR confers RI specificity to dual-specificity AKAPs, such as Ezrin (27), and this was further confirmed in the SPR studies because a RISR peptide partially disrupted the type I PKA-SFRS17A interaction but not the interaction with type II PKA.

SFRS17A was present in all cell lines tested, as revealed by reverse transcription-PCR, indicating that the protein is ubiquitously expressed in line with what has previously been reported regarding this protein being a housekeeping gene (46). The antibody characterized in this work was able to detect endogenous SFRS17A, which simplifies the characterization of this protein compared with previous reports that have based their conclusions on overexpression of SFRS17A. It also enabled us to do some of our experiments with the endogenous SFRS17A protein.

Proteins involved in pre-mRNA splicing are located in SFCs. It has been demonstrated that overexpressed SFRS17A colocalizes with the splicing factor SC35 in the nucleus (36), and in our study we obtained similar results from co-staining experiments
of endogenous SFRS17A and SC35. In addition, immunofluorescence experiments showed that SFRS17A colocalizes with the C subunit of PKA, placing all three proteins in SFCs. The C subunit was previously shown to be involved in regulation of pre-mRNA splicing (29) along with SFRS17A (36), SC35 (32), and numerous other proteins. Using an in vivo splicing assay, we demonstrated that SFRS17A had a significant effect on the alternative splice site selection of the adenoviral E1A gene. Furthermore, we also observed a similar splicing pattern for SFRS17A and the C subunit of PKA with increased expression of the two mRNAs of 10 and 9 S, whereas the larger mRNA product, 13 S, was reduced when compared with vector control. These findings indicate that SFRS17A promotes distal 5′ splice site selection. In contrast, introduction of SFRS17A, substituted to abolish type I PKA binding, did not confer the same effect on splicing, although it did appear to have some PKA-independent effect on the splicing pattern. Hence, we concluded that SFRS17A is involved in regulation of pre-mRNA splicing but that type I PKA, anchored through SFRS17A, appears to be necessary for activation of the distal 5′ splice site.

Several models may be envisioned for how PKA can regulate pre-mRNA splicing. The most obvious model would be that PKA affects splicing directly by binding to the AKAP SFRS17A and thereby being positioned to phosphorylate nearby substrates in the splicing factor compartments. It has, in fact, earlier been shown that PKA can phosphorylate the SR protein ASF/SF2 in vitro (47) and the polypyrimidine tract-binding protein in vivo (48). Studies have, however, been conducted that suggest pre-mRNA splicing is regulated by PKA through a cAMP-independent mechanism (29). The latter finding implies that the PKA R2C2 complex would not have to be activated by cAMP and could remain as an intact holoenzyme while regulating this process. Furthermore, PKA may recruit other proteins that have a regulatory role in the pre-mRNA splicing process. However, overexpression of the R subunits alone did not seem to influence the splicing pattern of the E1A minigene (data not shown), and further studies are needed to unravel the exact role of PKA in pre-mRNA splicing.

Although it is established that SFRS17A is a nuclear protein, the occurrence of R subunits in the nucleus remains elusive. However, some reports have been made that suggest the identification of R subunits in the nucleus (49–55). The uncertainty in this field may be based on the possibility that a nuclear R subunit is of low abundance and/or may only enter the nucleus when the nuclear envelope is disassembled at mitosis, leaving small pools of PKA associated with distinct AKAPs. Furthermore, we explored the possibility that SFRS17A could bind directly to the C subunit of PKA, but SPR studies, performed with C in the absence of R, did not show any interaction with SFRS17A; nor did C overlay on SFRS17A peptide arrays identify any interaction (data not shown). However, SPR studies performed with the intact PKA holoenzyme did show interaction, indicating that the R subunit is required for the interaction with SFRS17A (data not shown). Another intriguing possibility could be that SFRS17A shuttles in and out of the nucleus like its interaction partner ASF/SF2 (56) and carries PKA as cargo. However, preliminary immunofluorescence experiments using leptomycin B to inhibit active nuclear export and a combination of actinomycin D and cycloheximide to terminate ongoing transcription needed for nuclear reimport have not been able to confirm this. In summary, our data show that SFRS17A is a dual-specificity AKAP that targets PKA to the SFCs to confer PKA regulation of pre-mRNA splicing through a yet unknown mechanism.

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