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Cloning and Characterization of an Alternatively Spliced Form of SR Protein Kinase 1 That Interacts Specifically with Scaffold Attachment Factor-B*

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Eleni Nikolakaki‡§, Rachel Kohen¶, Annette M. Hartmann‖, Stefan Stamm, Elena Georgatsou¶, and Thomas Giannakouros‡**

*From the ‡Laboratory of Biochemistry, School of Chemistry, The Aristotelian University of Thessaloniki, Thessaloniki 54006, Greece, the ¶Laboratory of Biochemistry, Medical School, University of Thessaly, Larissa 41222, Greece, the ‖Ludwig Maximilian University, Nussbaumstrasse 7, München 80336, Germany, and the **Institute of Biochemistry, University of Erlangen-Nurenberg, Fahrstrasse 17, Erlangen 91054, Germany*

Serine/arginine protein kinases have been conserved throughout evolution and are thought to play important roles in the regulation of mRNA processing, nuclear import, germline development, polyamine transport, and ion homeostasis. Human SRPK1, which was first identified as a kinase specific for the SR family of splicing factors, is located on chromosome 6p21.2-p21.3. We report here the cloning and characterization of SRPK1a, which is encoded by an alternatively processed transcript derived from the *SRPK1* gene. SRPK1a contains an insertion of 171 amino acids at its NH_2-terminal domain and is similar to SRPK1 in substrate specificity and subcellular localization. Moreover, both isoforms can induce alternative splicing of human tau exon 10 in transfected cells. Using the yeast two-hybrid assay, we found that the extended NH_2-terminal domain of SRPK1a interacts with Scaffold Attachment Factor-B, a nuclear scaffold-associated protein. Confirmation of this interaction was provided by *in vitro* binding assays, as well as by co-immunoprecipitation from 293T cells doubly transfected with SRPK1a and SAF-B. Our studies suggest that different SRPK family members are uniquely regulated and targeted and thus the multiple SRPK kinases present in higher eukaryotes may perform specialized and differentiable functions.

Serine/arginine (SR[1]) protein kinases represent a novel class of enzymes that specifically modify SR or RS dipeptide motifs. To date, at least nine distinct genes encoding SR protein kinases have been identified in the genomes of mammals (*human SRPK1*, GenBank™ accession number U09564; *human SRPK2*, U88666A; *mouse SRPK1*, AJ224115; *mouse SRPK2*, B006036), yeast (*Saccharomyces cerevisiae Sky1*, S55098; *Schizosaccharomyces pombe Dsk1*, D13447), fruit fly (*Drosophila SRPK1*, AF01149), nematode (*Caenorhabditis elegans SPK-1*, AF241656), and plants (*Arabidopsis thaliana SRPK1*, AJ292978).

Mammalian SRPK1 and SRPK2, which are highly related in sequence, kinase activity, and substrate specificity, were initially purified and cloned on the basis of their ability to phosphorylate members of the SR family of splicing factors *in vitro* and mediate splicing factor redistribution during the cell cycle (1–4). SR proteins themselves constitute a highly conserved protein family that is intimately involved in the regulation of pre-mRNA splicing and other steps of RNA metabolism (for reviews, see Refs. 5–7). Biochemical studies demonstrated that SR proteins are required at multiple steps in the assembly of the spliceosome, the dynamic RNA-protein complex that catalyzes intron removal (8–10). Because RS domains are known to participate in protein-protein and protein-RNA interactions during spliceosome assembly, phosphorylation of these domains can modulate interactions involving SR proteins and is, therefore, essential for their function in constitutive splicing (3, 11, 12). Furthermore, phosphorylation of SR proteins leads to their release from nuclear speckles, in which they are concentrated to active sites of transcription in the nucleoplasm (1, 3, 13–15). Because changes in the intranuclear SR protein concentration play a critical role in determining which of the competing splice sites are selected, phosphorylation can also indirectly control alternative splice site selection (16–20). Finally, it has been proposed that the formation of complexes between SF2/ASF and SRPKs may modulate the subcellular distribution of SF2/ASF (21).

Yet, the lack both of authentic SR proteins in the yeast genome and of alternative mRNA splicing in yeast suggests that these kinases play roles in the regulation of cellular processes in addition to that of mRNA splicing. Indeed, genetic analyses have implicated Dsk1, which is the fission yeast homologue of SRPK1 in the regulation of chromosome segregation at the metaphase/anaphase transition (22). Furthermore, one of the endogenous substrates of Sky1p, in *S. cerevisiae*, is the RNA binding protein Npl3p, which has been implicated in mRNA transport (23). Sky1p was found to regulate nuclear import of Npl3p by promoting the interaction between Npl3p

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EBI Data Bank with accession number(s) AJ318054 and AJ224115.

§ To whom correspondence should be addressed: Laboratory of Biochemistry, School of Chemistry, Aristotelian University of Thessaloniki, Thessaloniki 54006, Greece. Tel.: 30-31-997726; Fax: 30-31-997689; E-mail: nikol@ccf.auth.gr.

[1] The abbreviations used are: SR, serine/arginine; SRPK serine/arginine protein kinase; SC, synthetic complete; LBR, lamin B receptor; SAF-B, scaffold attachment factor-B; S/MAR, scaffold/matrix attachment regions; bp, base pair(s); RT-PCR, reverse transcription-polymerase chain reaction; GST, glutathione *S*-transferase; CMV, cytomegalovirus; GFP, green fluorescence protein; AT, 3-aminotriazole; PMSF, phenylmethylsulfonyl fluoride; PAGE, polyacrylamide gel electrophoresis.

and its nuclear import receptor Mtr10p (24). In a recent report it was also demonstrated that Sky1p is a key regulator of polyamine transport in *S. cerevisiae* (25). *Sky1* disruption in yeast cells resulted in dramatically reduced uptake of spermine, spermidine, or putrescine. Data presented in the same report suggest that Sky1p is also involved in regulating ion homeostasis.

Further support for the hypothesis that SRPKs have a broader regulatory role was obtained with the finding that the *C. elegans* family member *spk-1* is predominantly expressed in germ cells and is required for germline development in *C. elegans* (26). In line with the above observations we have recently shown that SRPK1 is highly expressed in human and mouse testis and that the enzyme phosphorylates protamine 1 in an efficient and highly specific manner (27). SRPK1 also has other additional substrates, besides the SR family of splicing factors, such as the Lamin B Receptor (LBR), an integral protein of the inner nuclear membrane, that contains a stretch of RS repeats at its nucleoplasmic NH_2-terminal domain (28, 29).

Here we report the cloning and characterization of a novel *SRPK1* gene product, named SRPK1a, that contains an insertion of 171 amino acids at its NH_2-terminal domain. The two SRPK isoforms are nearly identical in terms of substrate specificity and subcellular localization and in their ability to induce alternative splicing in transfected cells. Furthermore, they were similarly expressed in various human tissues, although the level of expression of SRPK1a was significantly lower than that of SRPK1. Yet, using the yeast two-hybrid assay we found that, unlike SRPK1, SRPK1a interacts, via its NH_2-terminal domain, with SAF-B, a well characterized S/MAR binding protein. The so-called S/MARs (for Scaffold/Matrix Attachment Regions) are specialized DNA elements that mediate the attachment of chromatin to the nuclear scaffold and have been found in all eukaryotic organisms investigated (for review see Ref. 30). The implications of this interaction are discussed below.

MATERIALS AND METHODS

Cloning of Human SRPK1a—A Lambda ZAP Express human testis cDNA library (Stratagene, La Jolla, CA) was screened using a 617-bp fragment, comprising part of the sequence of human *SRPK1* (1) (nucleotides 262–879, starting from ATG; for details see Ref. 27). 5×10^5 plaques were screened by hybridization of Hybond filters (Amersham Pharmacia Biotech) using standard procedures (31) and yielded ten positive clones. One of the clones corresponded to full-length SRPK1. Two of the clones were identical to SRPK but contained an insertion of 513 bp between AAA (encoding lysine, the fourth amino acid of SRPK1) and GTG (encoding valine, the fifth amino acid of SRPK1). The sequence of both strands was determined using specific oligonucleotide primers and by a series of nested deletions and unidirectional exonuclease III digestion according to the manufacturer's instructions (*double-stranded* Nested deletion kit, Amersham Pharmacia Biotech, Uppsala, Sweden). The EMBL data bank accession number for SRPK1a is AJ318054.

Northern Blot Hybridization—Human multiple tissue I and II Northern blot filters were purchased from CLONTECH Laboratories (Palo Alto, CA) and processed as previously described (27). A cDNA of 860 bp, comprising nucleotides 1–860 of the sequence of mouse *SRPK1* (EMBL data bank accession number AJ224115; see also Ref. 27), and a PCR fragment of 528 bp, comprising nucleotides 1–528 of the coding region of human *SRPK1a*, were used as probes for *SRPK1* and *SRPK1a*, respectively. The 860-bp mouse fragment is 92% identical, at the DNA level, (98% at the protein level) to the respective human fragment.

RT-PCR Analysis—RT-PCR analysis was performed using the Titan One Tube RT-PCR system (Roche Molecular Biochemicals, Mannheim D-68298, Germany) as per the manufacturer's instructions. Total RNA from human testis, provided from CLONTECH laboratories, was used as template. Two micrograms of testis RNA was initially denatured at 94 °C for 1 min and then incubated, for first-strand cDNA synthesis, with 10 pmol of antisense primer (5′-CTTCCTGCTCTGGTAGATCAC-3′) at 55 °C for 35 min. The following sense primer (10 pmol) was used for PCR amplification: 5′-GGTCTCACCATGGAGCGGAAA-3′. PCR conditions were: denaturation at 94 °C for 2 min, followed by 40 cycles: 30-s denaturation at 94 °C, annealing at 58 °C for 30 s, extension at 68 °C for 40 s, and a final extension at 68 °C for 10 min. Amplified products were resolved by electrophoresis through 1% agarose gel and ethidium bromide staining.

Plasmids, Expression of Proteins, and Antibodies—The pGEX-2T bacterial expression vector (Amersham Pharmacia Biotech) was used to express human SRPK1 (27, 28) and the NH_2-terminal domain of SRPK1a (amino acids 1–176; construct termed GST-SRPK1aNt) fused with glutathione *S*-transferase (GST) in *Escherichia coli*. To subclone the NH_2-terminal domain of *SRPK1a* into pGEX-2T, two primers (sense: 5′-AGTAGGATCCATGGAGCGGAAAGGTGAGCGG-3′, containing a *Bam*HI site; antisense: 5′-CTAGAAGCTTCACTGCAGGAGAGAGGGATGG-3′) were used to amplify a DNA fragment, comprising nucleotides 1–528 of the coding region of *SRPK1a*, by polymerase chain reaction. PCR was performed as described (29) but with the addition of 10% Me_2SO. The PCR fragment was digested with *Bam*HI, repurified, and cloned into the *Bam*HI and *Sma*I sites of pGEX-2T. Wild-type and specifically altered forms of LBR protein were prepared as GST fusion proteins as previously described (29). Briefly, wtNt: contains the NH_2-terminal domain of LBR (amino acids 1–205); ΔRSNt: contains the NH_2-terminal domain of LBR but lacks the RS motifs (amino acids 75–84; ^{75}RSRSRSRSRS^{84}); wtNtG^{78}, wtNtA^{80}, wtNtA^{82}, and wtNtA^{84}: fusion proteins identical to wtNt except that in each case Ser^{78}, Ser^{80}, Ser^{82}, or Ser^{84} was mutated to Gly or Ala. Full-length SRPK1 and SRPK1a were also subcloned into the p-FLAG-CMV-2 (Eastman Kodak) vector and expressed in 293T cells with a FLAG tag fused at their NH_2 termini. For this purpose oligonucleotides corresponding to the 5′- and 3′-complementary coding regions of human *SRPK1* and *SRPK1a* with additional *Eco*RI sites at the 5′- and 3′-ends, respectively, were prepared (*SRPK1*, sense: 5′-CGGGAATTCTATGGAGCGGAAAGTGCTTGCG-3′, antisense: 5′-CGAGAATTCCCGGAGTTAAGCCAAGGGTGCCG-3′; *SRPK1a*, sense: 5′-CGGGAATTCTATGGAGCGGAAAGGTGAGCGG-3′, antisense: 5′-CGAGAATTCCCGGAGTTAAGCCAAGGGTGCCG-3′), and PCR was performed. The products were digested with *Eco*RI, repurified, and cloned into the *Eco*RI site of p-FLAG-CMV-2 in the correct orientation. Rat SAF-B-PC encoding the COOH-terminal part of rat SAF-B (starting from nucleotide 1746 of the rat SAF-B sequence; EMBL data bank accession number AF056324) was expressed as a GST fusion protein or subcloned into the pEGFP-C2 vector (CLONTECH) and expressed in 293T cells with green fluorescence protein (GFP) fused at its NH_2 terminus as previously described (32).

Polyclonal antibodies against SRPK1/SRPK1a and specifically against SRPK1a were produced by injecting GST-SRPK1 and GST-SRPK1aNt into rabbits as described previously (33). The M5 anti-FLAG monoclonal antibody was a kind gift of George Mosialos (Biomedical Sciences Research Center Al. Fleming, Vari, Attiki), whereas the anti-GFP monoclonal antibody was obtained from Roche Molecular Biochemicals.

Yeast Two-hybrid Screen—A 528-bp fragment coding for the NH_2-terminal domain of SRPK1a (SRPK1aNt; see above) was amplified by PCR (sense primer: 5′-CTGGAATTCATGGAGCGGAAAGGTGAGCGG-3′; antisense primer: 5′-CTAGAAGCTTCACTGCAGGAGAGAGGGATGG-3′), digested with *Bam*HI, and cloned into the *Bam*HI and *Sma*I sites of pGBT9 (CLONTECH) in-frame with the GAL4 DNA binding domain. *S. cerevisiae* strain pJ69-4A (*MAT***a** *trp1–901 leu2–3, 112 ura3–52 his3–200 gal4*Δ *gal80*Δ *LYS2*::*GAL1-HIS3 GAL2-ADE2 met2*::*GAL7-lacZ*) (34) was co-transformed with pGBT9-SRPK1aNt and an equimolar mixture of E9.5 and E10.5 mouse embryonic cDNA libraries constructed in the pVP16 fusion vector (35). Positive clones were selected on SC (synthetic complete) minus Trp, Leu, His medium (36) containing 11.25 μM Ade and 3 mM AT (3-amino triazole). The positive pVP16-derived plasmids isolated in this screen were rescued and used to again co-transform yeast strain pJ69-4A with pGBT9-SRPK1aNt or pGBT9-SRPK1 to confirm and test the specificity of the interactions. The coding region of SRPK1 was amplified by PCR (sense primer: 5′-GTAGAATTCATGGAGCGGAAAGTGCTTGCG-3′; antisense primer: 5′-TAGGAATTCGGAGTTAAGCCAAGGGTGCCG-3′), digested with *Eco*RI, and cloned into the *Eco*RI site of pGBT9 in the correct orientation.

Cell Culture, Western Blotting, and Immunoprecipitation—Human 293T cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% (v/v) fetal calf serum and antibiotics. 5×10^5 cells (~50% confluent) were transfected with 5 μg of p-FLAG-CMV-2-SRPK1 or p-FLAG-CMV-2-SRPK1a DNA using the calcium phosphate method (37). Total amounts of plasmid DNA were made up to 20 μg with pcDNA3 (Invitrogen). After 16 h the medium was changed and the

cells were incubated for another 24 h. Cells were lysed with 200 μl of 1% Triton buffer (1% Triton X-100, 50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 10 μg/ml aprotinin, and 1 mM PMSF) for 30 min on ice. Whole cell extracts were clarified by centrifugation for 15 min in a microcentrifuge, and the protein concentration was determined by the method of Bradford (38). One hundred and fifty micrograms of each lysate was supplemented with the appropriate volume of 5× sample electrophoresis buffer and analyzed on 10% SDS-PAGE. Western blotting was performed with the M5 anti-FLAG monoclonal antibody, an alkaline phosphatase-coupled goat anti-mouse secondary antibody, and 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium substrate. For immunoprecipitation 100 μg of each extract was incubated with 1 μl of the M5 anti-FLAG monoclonal antibody or with 5 μl of the anti-SRPK1 or the anti-SRPK1a polyclonal antibodies for 3 h on ice. Twenty microliters of protein G beads (for the monoclonal antibody) or protein A beads (for the polyclonal antibodies) was added and incubated overnight on ice. Antigen-antibody complexes were collected by centrifugation and were washed three times with lysis buffer. *In vitro* phosphorylation assays were performed on beads as described previously (29) using as substrates wild-type and specifically altered forms of LBR protein (see above) and recombinant ASF/SF2 (kindly provided by Jamal Tazi, Institut de Génétique Moléculaire de Montpellier, UMR 5535, CNRS). The same procedure was followed for immunoprecipitation from rat testis cytosol.

Cells co-transfected with plasmid DNA encoding GFP-SAF-B-PC and FLAG-SRPK1 or FLAG-SRPK1a were lysed in 200 μl of radioimmune precipitation buffer (1% Nonidet P-40, 1% sodium deoxycholate, 0.1% SDS, 150 mM NaCl, 10 mM sodium phosphate, pH 7.2, 2 mM EDTA, 10 μg/ml aprotinin, and 1 mM PMSF). One hundred microliters of the supernatants, following centrifugation, was diluted 4-fold in radioimmune precipitation rescue buffer (10 mM sodium phosphate, pH 7.2, 20 mM NaCl, 10 μg/ml aprotinin, and 1 mM PMSF) and immunoprecipitations were performed overnight at 4 °C, under shaking, using 3 μl of anti-GFP antibody and 20 μl of protein G-Sepharose. The beads were washed three times in a buffer (50 mM Hepes, pH 7.5, 150 mM NaCl, 1 mM EDTA, 10% glycerol, 0.1% Triton X-100, 10 μg/ml aprotinin, and 1 mM PMSF) and resuspended in 30 μl of SDS sample buffer. The bound proteins were subsequently analyzed on 10% SDS-PAGE followed by Western blotting using the M5 anti-FLAG monoclonal antibody.

GST Pull-down Experiments—GST-SAF-B (~3–4 μg) immobilized on glutathione-Sepharose beads was incubated with 50 μl of cell extract, derived from 293T cells transfected with plasmid DNA encoding either FLAG-SRPK1 or FLAG-SRPK1a, in phosphate-buffered saline buffer (20 mM phosphate buffer, pH 7.4, 150 mM NaCl, 0.5 mM PMSF) in a total volume of 0.5 ml. Incubations were carried out for 60 min at room temperature. The beads were harvested, washed three times with phosphate-buffered saline, and resuspended in 25 μl of SDS sample buffer. Bound SRPK1 and SRPK1a were analyzed on 10% SDS-polyacrylamide gels and detected by Western blotting using the M5 anti-FLAG monoclonal antibody.

In Vivo Splicing of Human Tau 9-10-11 Minigene—Tau construct SV9/10/11, containing exon 10 flanked by tau exons 9 and 11, was described earlier (39). *In vivo* splicing was performed essentially as described (40). Briefly, 3×10^5 HEK293 cells were transfected with 1 μg of the reporter gene together with increasing amounts (0, 0.5, and 1 μg) of plasmid DNA encoding FLAG-SRPK1 or FLAG-SRPK1a, using the calcium phosphate method. RNA was isolated 17–24 h following transfection, using the RNeasy mini kit (Qiagen), according to the manufacturer's instructions. Reverse transcription and PCR were carried out as described previously (40).

RESULTS

In our previous reports we used a PCR approach to reclone the *SRPK1* cDNA to examine its relationship with a previously identified LBR kinase (28, 29) and identify novel substrates besides the SR family of splicing factors (27). In this respect we screened a Lambda ZAP Express human testis cDNA library, at high stringency, using as a probe a 617-bp PCR fragment comprising nucleotides 262–879 of the sequence of human *SRPK1* (for details see Ref. 27). We isolated three full-length cDNA sequences corresponding to *SRPK1*. However, one of the clones contained an insertion of 513 bp between AAA (encoding lysine, the fourth amino acid of SRPK1) and GTG (encoding valine, the fifth amino acid of SRPK1), which is absent from the known sequence of *SRPK1* (see Figs. 1 and 2). Because it is well

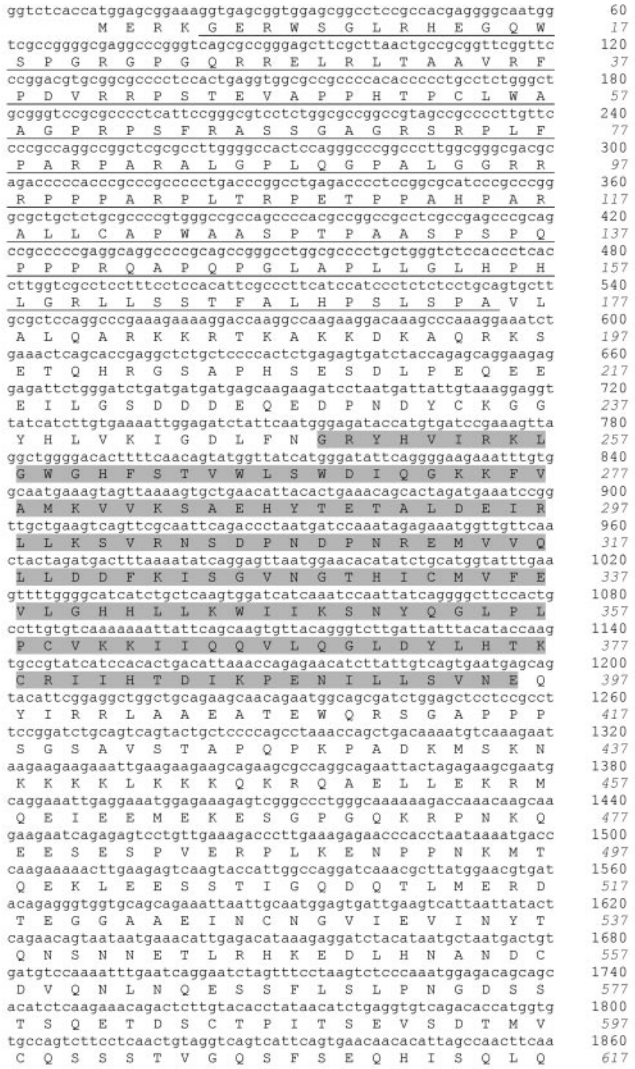

FIG. 1. **Nucleotide and predicted amino acid sequences of SRPK1a (EMBL accession number AJ318054).** *Underlined* is the sequence encoded by the 513-bp insert, and *shaded* are kinase catalytic domains. A spacer sequence separates the catalytic domains.

documented that human *SRPK1* is mapped to chromosome 6p21.2-p21.3 (41) and the genomic sequence is available (EMBL data bank, locus HS422H11, accession number Z99128) we compared the sequence of the isolated clone with the genomic sequence. The sequence alignment revealed that the *SRPK1* gene comprises 16 exons (Fig. 2*A*, see also EMBL data bank, locus HS422H11, accession number Z99128, gene = dJ422H11.1). The 513-bp insert is located between the first two exons of *SRPK1*. This suggests that this segment may be alternatively spliced. The *SRPK1* cDNA originally isolated by

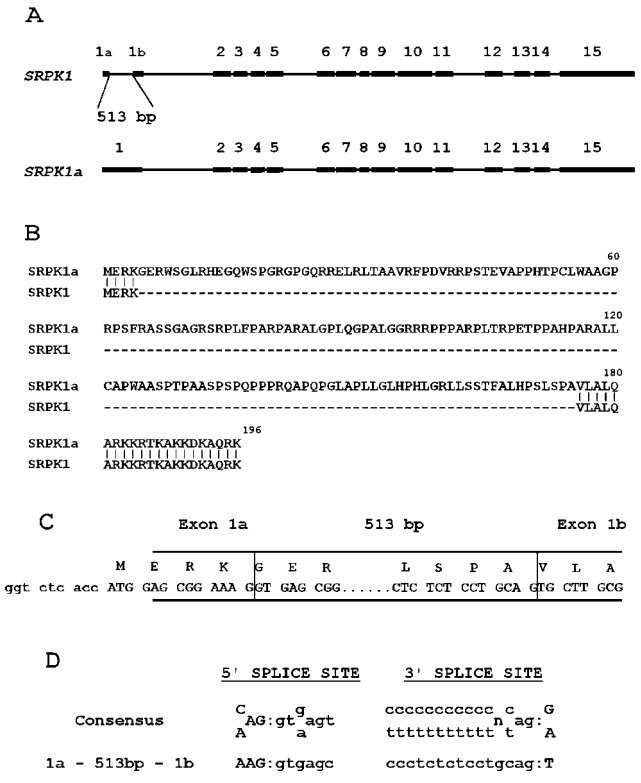

FIG. 2. **Schematic representation of the alternative splicing of the 513-bp fragment.** *A*, schematic representation of the *SRPK1a*/*SRPK1* gene structure. Sequence comparison of AJ318054 and Z99128 revealed the genomic organization of the *SRPK1a*/*SRPK1* gene. *Boxes* indicate exons; nucleotide positions in Z99128 are 94672–94651, 94136–94076, 64621–64503, 62531–62423, 61719–61632, 60419–60332, 47947–47841, 46336–46171, 44567–44542, 44102–43889, 43509–43090, 42718–42621, 30976–30869, 16212–16143, 12037–11945, 9096–6642 (the genomic sequence is antiparallel to AJ318054). Exon 1 within the *SRPK1a* transcript comprises exons 1a and 1b of SRPK1 as well as the 513-bp segment (nucleotides 94650–94137), which is excluded from *SRPK1*. *B*, comparison of the amino acid sequence encoded by exon 1 of SRPK1a with the respective sequences encoded by exons 1a and 1b of SRPK1. The sequence encoded by the 513-bp segment, which is absent in SRPK1, is denoted by the *dashed line*. *C*, the nucleotide sequence surrounding the alternatively spliced 513-bp segment. Splicing out of the 513-bp segment would result in production of SRPK1 (containing the amino acid sequences encoded by exons 1a and 1b). Splicing in of the 513-bp segment would result in production of SRPK1a (containing the amino acid sequence encoded by the entire exon 1). *D*, 5′- and 3′-splice sites found at the boundaries between exons 1a/1b and the 513-bp segment. The coding sequence in both *SRPK1a* and *SRPK1* is *capitalized*, whereas the spliced out sequence (513-bp segment) from *SRPK1* is in *lowercase letters*.

Gui *et al.* (1) would encode the isoform lacking the 171 amino acids encoded by the 513-bp segment, whereas the novel cDNA, designated *SRPK1a* (EMBL data bank, accession number AJ318054), would encode the isoform containing this sequence (Fig. 2, *A–C*). Further analysis revealed the presence of 5′- and 3′-splice sites at the ends of the intervening regions, suggesting that the 513-bp segment normally represents an intronic sequence that is not, however, spliced out from the *SRPK1a* transcript (Fig. 2*D*).

The extended NH_2-terminal domain of SRPK1a shares no significant similarity to known proteins and contains a relatively high number of proline residues (Fig. 1*B*). Yet, unlike SRPK2, it does not contain the PPLP consensus motif required for the binding of a subclass of WW domain-containing proteins (3, 42). Most notably, SRPK1a contains two L*XX*LL motifs (^{148}LAPLL^{152} and ^{158}LGRLL^{162}), which are thought to facilitate the interaction of different proteins with nuclear receptors (43, 44).

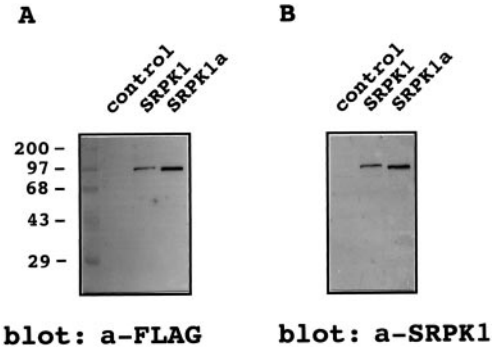

FIG. 3. **Expression of SRPK1a in mammalian cells.** Lysates from 293T cells transfected with the pCMV-2 vector alone (*control*) or with pCMV-2 vectors encoding FLAG-tagged SRPK1 or SRPK1a were analyzed on 10% SDS-polyacrylamide gels. The proteins were then transferred to nitrocellulose, and epitope-tagged SRPK1 or SRPK1a were detected with the M5 anti-FLAG monoclonal antibody (*A*) or the anti-SRPK1 polyclonal antibody (*B*).

The predicted molecular mass of the kinase encoded by the *SRPK1a* cDNA is 92.3 kDa. To detect this protein product, we tagged the SRPK1a polypeptide with a FLAG epitope and expressed the fusion protein in 293T cells. As a control we used FLAG-tagged SRPK1. Extracts from 293T cells transfected with the pCMV-2 vector alone showed no proteins detected by anti-FLAG immunoblotting (Fig. 3*A*, control), whereas extracts from 293T cells transfected with pCMV-2 vectors encoding FLAG-tagged SRPK1 or SRPK1a demonstrated immunoreactive proteins of ~97 and 92 kDa, respectively (Fig. 3*A*; note that, although the predicted molecular mass of SRPK1 is 74.3 kDa, the protein was shown to migrate on SDS-polyacrylamide gels with an apparent molecular mass of 92–95 kDa; see Refs. 1 and 21). The same bands were also obtained when the immunoblotting was performed with an anti-SRPK1 polyclonal antibody raised against GST-SRPK1 (Fig. 3*B*). Only a faint band of 92 kDa corresponding to FLAG-SRPK1a could be detected when the immunoblotting was performed with a specific anti-SRPK1a polyclonal antibody raised against GST-SRPK1aNt (the extended NH_2-terminal domain of SRPK1a fused to GST; data not shown).

To demonstrate that *SRPK1a* cDNA encodes an active protein kinase, we carried out immunoprecipitation experiments. FLAG-tagged SRPK1 or SRPK1a were transiently transfected into 293T cells, and the cells were harvested and lysed 48 h post-transfection. Immunoprecipitations were carried out with either the M5 anti-FLAG monoclonal antibody or the anti-SRPK1 polyclonal antibody or the specific anti-SRPK1a polyclonal antibody. SR protein-kinase activity was assayed on beads incubated in kinase buffer with bacterially expressed ASF/SF2 or LBR as substrates and radiolabeled ATP. As shown in Fig. 4 the anti-FLAG and the anti-SRPK1 antibodies immunoprecipitated both SRPK1 and SRPK1a, whereas the specific anti-SRPK1a antibody immunoprecipitated a kinase activity only from 293T cells transfected with pCMV-2 vector encoding FLAG-tagged SRPK1a. Interestingly, we reproducibly observed that SRPK1a was 2- to 3-fold more active than SRPK1. As expected, SRPK1a-mediated phosphorylation took place in the RS domain, because the immunoprecipitated enzyme was unable to modify a GST fusion protein that contains the NH_2-terminal domain of LBR but lacks the RS motifs (Δ*RSNt*, Fig. 4). Furthermore, as previously shown with SRPK1 (28), SRPK1a efficiently phosphorylated various derivatives of the NH_2-terminal domain of LBR fused to GST, in which Ser^{78}, Ser^{80}, Ser^{82}, and Ser^{84} of the RS domain (^{75}RSRSRSRSRS^{84}) were mutated to Gly (wtNtG^{78}), Ala

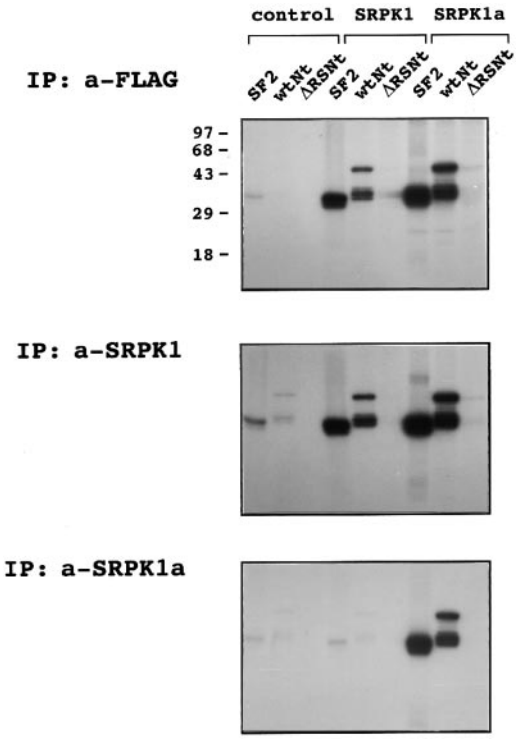

FIG. 4. **SRPK1a cDNA encodes an active protein kinase.** 293T cells were transiently transfected with the pCMV-2 vector alone (*control*) or with pCMV-2 vectors encoding FLAG-tagged SRPK1 or SRPK1a. Extracts were prepared at 48 h post-transfection, and immunoprecipitations were carried out with either the M5 anti-FLAG monoclonal antibody or the anti-SRPK1 polyclonal antibody or the specific anti-SRPK1a polyclonal antibody. The immunoprecipitates were incubated under *in vitro* phosphorylation conditions with bacterially produced SF2, wtNt (a GST fusion protein containing the NH_2-teminal domain of LBR; amino acids 1–205) and ΔRSNt (contains the NH_2-teminal domain of LBR but lacks the RS motifs; amino acids 75–84). Labeled proteins were detected by SDS-PAGE and autoradiography. The full-length wtNt migrates with an apparent molecular mass of ~51 kDa. The *lower bands* represent degradation products (see also Ref. 29). The gels were exposed for 2 h. *Bars* on the *left* indicate molecular masses (in kDa).

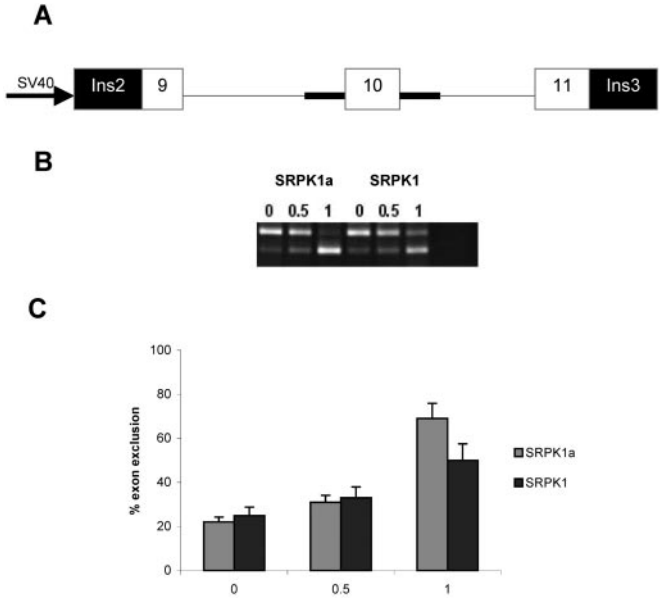

FIG. 5. **Influence of SRPK1a and SRPK1 on alternative splicing of human tau exon 10.** *A*, schematic representation of the expression construct SV9/10L/11. Exon 10 is flanked by tau exons 9 and 11. Exons 9 and 11 are fused with insulin exons 2 and 3, respectively. Primers located in these insulin exons were used for amplification. *Thick lines* around exon 10 indicate the flanking intronic regions (471 nucleotides downstream, 409 nucleotides upstream). The SV40 promoter is indicated with an *arrow*. *B*, HEK293 cells were co-transfected with 1 μg of the SV910L/11 minigene together with increasing amounts (0, 0.5, and 1 μg) of plasmid DNA encoding FLAG-SRPK1 or FLAG-SRPK1a. RNA was isolated 17–24 h following transfection, and RT-PCR was carried out. The splice products have a size of 336 and 429 nucleotides, respectively. *C*, statistical evaluation of three independent experiments. *Numbers* indicate micrograms of SRPK1 or SRPK1a expression constructs transfected.

(wtNtA^{80}), Ala (wtNtA^{82}), and Ala (wtNtA^{84}), respectively (data not shown).

Next, we determined the subcellular localization of FLAG-tagged SRPK1 and 1a in transfected COS-1 cells by indirect immunofluorescence using the M5 anti-FLAG monoclonal antibody. Consistent with previous reports for SRPK1 (2, 3) the cytoplasmic signal was predominant for both isoforms, although the expressed kinases were also clearly visible in the nucleus (data not shown). The cytoplasmic and nuclear localization is a characteristic feature of all SRPK family members and probably reflects a function for these kinases in both compartments. An alternative, although not mutually exclusive, hypothesis is that the cytoplasmic sequestering of these enzymes may regulate their nuclear functions by controlling their availability to nuclear substrates.

SR protein kinases affect alternative splicing through a phosphorylation-mediated release of SR proteins from nuclear storage sites. In this respect we have previously shown that SRPK1 was able to regulate the alternative splicing of human tau exon 10 in transfected cells (45). To determine whether SRPK1a had an effect similar to that of SRPK1, we tested both isoforms in co-transfection experiments with the tau minigene SV9–10L-11 consisting of exon 10 and its flanking exon and intron regions (Fig. 5*A*; see also Ref. 39). Without addition of SRPK1, exon 10 is skipped in about 25% of the minigene-derived RNA, whereas in the presence of SRPK1 or SRPK1a this percentage increases to about 50–70% (Fig. 5*B*). Consistent with our data from the immunoprecipitation experiments in transfected cells (see Fig. 4) SRPK1a was more efficient than SRPK1 in promoting skipping of human tau exon 10.

Thus both isoforms are very similar in terms of substrate specificity and subcellular localization and in their ability to induce alternative splicing in transfected cells. It is, however, unlikely that they are completely redundant kinases, because they may be differentially expressed and/or regulated. To explore these possibilities we first examined the expression of both isoforms, by Northern blotting analysis, in multiple human tissues as well as in various human cancer cell lines. In agreement with our previous report, *SRPK1* was predominantly expressed in the testis but was also present at low levels in most of the tissues examined (Fig. 6*A*; see also Ref. 27). *SRPK1a* was clearly detected only in testis, at a significantly lower percentage the level of *SRPK1* (Fig. 6*B*). For both isoforms we detected a smaller transcript of 2.7 kb in the testis (see also Ref. 27).

To further demonstrate that SRPK1a is expressed in testis we performed reverse transcription coupled with PCR amplification (RT-PCR) to amplify the region encompassing the 513-bp segment of *SRPK1a* (nucleotide position 1–658, see Fig. 1) from human testis-derived cDNA. Two DNA species were amplified, the sizes of which (145 and 658 bp, respectively) were consistent with alternative splicing of the 513-bp segment (Fig. 7*A*). In addition we carried out immunoprecipitation experiments from rat testis cytosol with the specific anti-SRPK1a polyclonal antibody or the anti-SRPK1 polyclonal antibody. As shown in Fig. 7*B* the specific anti-SRPK1a antibody immunoprecipitated a kinase activity that was able to phosphorylate ASF/SF2 or recombinant LBR but not ΔRSNt that lacks the RS

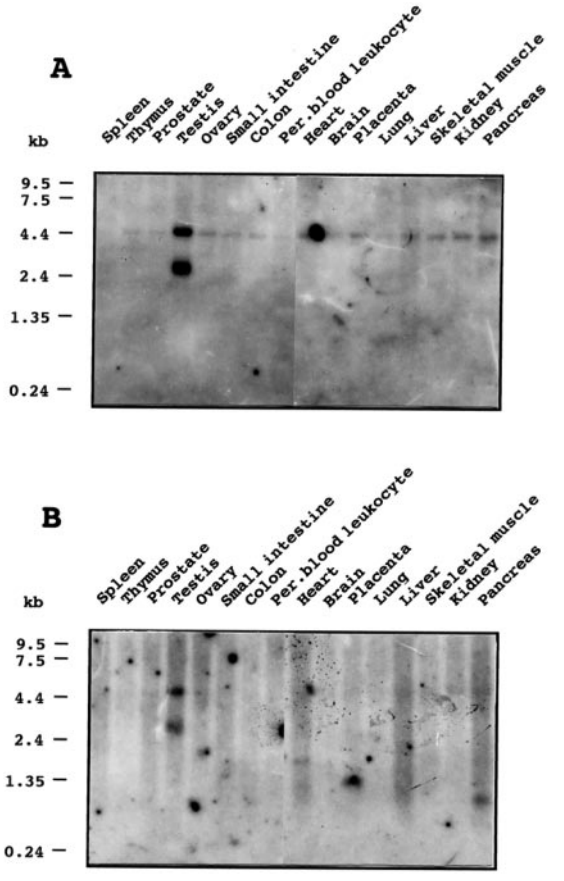

FIG. 6. **Northern blotting analysis of *SRPK1* and *SRPK1a*.** Human multiple tissue I and II Northern blot filters were purchased from CLONTECH and processed as described under "Materials and Methods." A cDNA of 860 bp, comprising nucleotides 1–860 of the sequence of mouse *SRPK1* (EMBL data bank accession number AJ224115; see also "Materials and Methods" and Ref. 27), and a PCR fragment of 528 bp, comprising nucleotides 1–528 of the coding region of human *SRPK1a*, were used as probes for *SRPK1* (*A*) and *SRPK1a* (*B*), respectively. Exposure time in *B* was seven times as long as in *A*. Positions of molecular size markers in kilobases, are indicated.

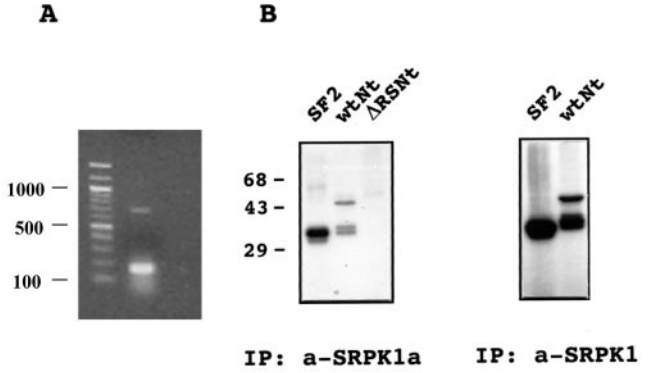

FIG. 7. **Expression of SRPK1a in human and rat testis.** *A*, RT-PCR analysis of *SRPK1a*/*SRPK1* transcripts demonstrates alternative splicing. Oligonucleotide primers flanking the 513-bp segment were used to amplify the cDNA generated from human testis total RNA. The spliced product has a predicted size of 145 bp, whereas the unspliced product, containing the 513-bp segment, has a predicted size of 658 bp. A 100-bp DNA ladder is shown on the *left*. *B*, SRPK1a or both SRPK1 and SRPK1a were immunoprecipitated from rat testis cytosol with the specific anti-SRPK1a polyclonal antibody or the anti-SRPK1 polyclonal antibody, respectively. The immunoprecipitates were incubated under *in vitro* phosphorylation conditions with SF2, wtNt, and ΔRSNt (SRPK1a only). Labeled proteins were detected by SDS-PAGE and autoradiography. The gels were exposed for 2 days. *Bars* on the *left* indicate molecular masses (in kDa).

motifs, whereas the anti-SRPK1 antibody immunoprecipitated a 4-fold higher kinase activity, corresponding to both SRPK1 and SRPK1a. The ratio of SRPK1/SRPK1a, as estimated from the immunoprecipitation experiment, is lower than the ratio observed with the RT-PCR and Northern blotting analyses. This is probably due to the higher activity of SRPK1a as compared with that of SRPK1.

Next, a yeast two-hybrid screen was performed to isolate proteins that interact specifically with the extended NH_2-terminal domain of SRPK1a, because it is well known that interactions through specific protein modules mediate the specificity of signal transduction events. To this end, *SRPK1aNt* (encoding amino acids 1–176) was subcloned to pGBT9 in-frame with the GAL4 DNA binding domain. The yeast strain pJ69-4A carrying the pGBT9-SRPK1aNt vector was transformed with an equimolar mixture of E9.5 and E10.5 mouse embryonic cDNA libraries in which cDNAs were fused to the coding sequence for the VP16 activation domain. Screening of $\sim 8 \times 10^6$ recombinant clones led to the isolation of about 80 that grew on the appropriate selection medium and gave detectable β-galactosidase activity. Twenty-one clones remained positive for β-galactosidase activity when co-transformed with the SRPK1aNt fusion protein but not with the DNA binding domain of GAL4 alone. As deduced by DNA sequencing and BLAST searching three of the positive clones, showing the strongest interaction, were different isolates of SAF-B, a nuclear scaffold-associated protein. On the basis of the SAF-B sequences isolated by the two-hybrid screen, COOH-terminal residues 585–720 (EMBL data bank accession number AF056324) appear to be sufficient for the interaction with the NH_2-terminal domain of SRPK1a. Most interestingly, the observed interaction was specific, because all three clones showed a very weak interaction with SRPK1 when pJ69-4A carrying the clones was co-transformed with a cDNA, encoding the full-length coding region of SRPK1, fused to the GAL4 binding domain (Fig. 8).

To confirm the interactions detected in the yeast two-hybrid assay, we showed that SRPK1a, but not SRPK1, bound strongly to SAF-B *in vitro* (Fig. 9*A*). Purified GST-SAF-B-PC (containing the COOH-terminal domain of SAF-B, amino acids 493–874; see also Ref. 32), or GST alone were incubated with cell extracts from 293T cells transfected with pCMV-2 vectors encoding either FLAG-tagged SRPK1 or SRPK1a. The complexes were recovered by pull-down with glutathione-Sepharose beads and analyzed by Western blotting with the M5 anti-FLAG monoclonal antibody. The specific interaction of SRPK1a with SAF-B was also demonstrated *in vivo* by co-immunoprecipitation/Western blotting analysis (Fig. 9*B*). To this end FLAG-tagged SRPK1a or SRPK1 and GFP-tagged SAF-B-PC were transiently transfected in 293T cells. Complexes between SAF-B and SRPKs were immunoprecipitated with an anti-GFP monoclonal antibody and separated by SDS-PAGE. The proteins were then transferred to nitrocellulose and epitope-tagged SRPK1a or SRPK1 were detected with the M5 anti-FLAG monoclonal antibody. When FLAG-tagged SRPK1a was co-transfected with GFP-tagged SAF-B-PC, the 92-kDa FLAG-SRPK1a polypeptide was detected in the anti-GFP immunoprecipitate (Fig. 9*B*, *last lane*), demonstrating a stable interaction between SAF-B and SRPK1a. When single transfections with FLAG-SRPK1a were carried out, the 92-kDa band was not obtained in the immunoprecipitates (Fig. 9*B*, *lane 1*), ruling out the possibility that the anti-GFP antibody directly immunoprecipitated SRPK1a.

These results strongly suggest that one of the SRPK family members binds specifically to a nuclear scaffold-associated protein. SAF-B has been found phosphorylated *in vivo* (46). How-

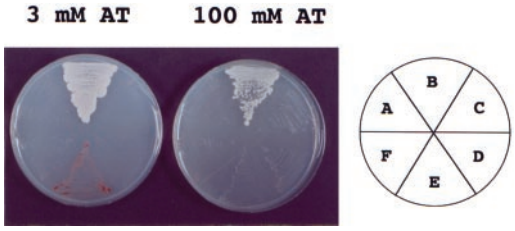

FIG. 8. **The NH_2-terminal domain of SRPK1a interacts with SAF-B in yeast.** *S. cerevisiae* strain pJ69-4A was co-transformed with (*A*) pGBT9-SRPK1aNt and pVP16, (*B*) pGBT9-SRPK1aNt and pVP16-SAF-B, (*C*) pGBT9 and pVP16-SAF-B, (*D*) pGBT9- SRPK1 and pVP16, (*E*) pGBT9-SRPK1 and pVP16-SAF-B, and (*F*) pGBT9 and pVP16. The strains were streaked in the same order on plates containing SC minus Trp, Leu, Ade, His, and 3 mM AT (*left*) or SC minus Trp, Leu, Ade, His, and 100 mM AT (*right*). The *SAF-B* clone used in these experiments encodes amino acids 585–720 of rodent SAF-B (EMBL data bank accession number AF056324).

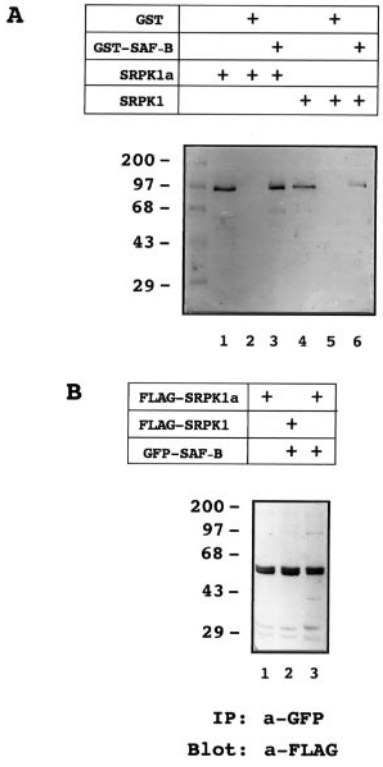

FIG. 9. **Interaction between SRPK1a and SAF-B *in vitro* and in mammalian cells.** *A*, GST-SAF-B-PC or GST alone, as a negative control, were incubated with cell extracts from 293T cells transfected with pCMV-2 vectors encoding either FLAG-tagged SRPK1a or SRPK1. The complexes were recovered by pull-down with glutathione-Sepharose beads and analyzed on 10% SDS-polyacrylamide gels. Bound SRPK1a or SRPK1 were detected by Western blotting using the M5 anti-FLAG monoclonal antibody. A standard amount of cell extract, one-third of which is shown (*lanes 1* and *4*), was used in each binding assay. *B*, 293T cells were co-transfected with plasmids expressing FLAG-tagged SRPK1a or SRPK1 and GFP-tagged SAF-B-PC. Complexes between SAF-B and SRPKs were immunoprecipitated with an anti-GFP monoclonal antibody and analyzed on 10% SDS-polyacrylamide gels. The proteins were then transferred to nitrocellulose, and epitope-tagged SRPK1a or SRPK1 were detected with the M5 anti-FLAG monoclonal antibody. No direct immunoprecipitation of FLAG-tagged SRPK1a by the anti-GFP antibody was observed (*lane 1*).

ever, this protein is not a potential substrate of the SRPKs, because it does not contain any alternating RS or SR dipeptides. Indeed, we have been unable to detect any phosphorylation of GST-SAF-B-PC by immunoprecipitated FLAG-tagged SRPK1a or bacterially expressed GST-SRPK1 (data not shown). This implies that SAF-B acts as a "docking site" rather than as a substrate for SRPK1a and thereby, RS domain-containing proteins, which are in close vicinity with the nuclear scaffold, may be preferentially targeted by this kinase isoform.

DISCUSSION

We report here the cloning and characterization of a third member of the SRPK family of protein kinases, which is an alternatively spliced form of SRPK1, named SRPK1a. The SRPK1/SRPK1a locus is situated on human chromosome 6. Splicing out of a 513-bp segment, which is located between exons 1a and 1b, would result in the production of SRPK1, whereas splicing in of the 513-bp segment would yield SRPK1a (see Fig. 2). In the latter case a large exon 1 is assembled within the *SRPK1a* transcript that comprises exons 1a and 1b of SRPK1 as well as the 513-bp segment.

Probably, SRPK1a does not represent the only paradigm of SR protein kinase regulation at the pre-mRNA processing level. Bedford *et al.* (47) identified a partial mouse cDNA clone, WBP6 (WW-domain binding protein 6; accession number U92456), based on its binding to a WW domain-containing protein probe in an expression screen. The nucleotide sequence of this clone is identical to mouse SRPK2 with the exception of an additional NH_2-terminal sequence in WBP6. Although further studies are required, this suggests that WBP6 may be an alternatively spliced SRPK2 product found in the mouse. Furthermore, two more isoforms encoded by the *SRPK1* gene, each containing a different exon 14, have been predicted by the Sanger Center (locus HS422H11, accession number Z99128, gene products dJ422H11.1.1 and dJ422H11.1.2). Exon 14 encodes the amino acid sequence between catalytic subdomains X and XI (catalytic subdomains are indicated analytically in Ref. 1). Even though the existence of those isoforms is not experimentally documented, our data, supported by the above prediction, raise the intriguing possibility that four different isoforms may arise from a single *SRPK1* gene.

Several isoforms of the ubiquitous LAMMER protein kinase family (named upon the existence of the motif EHLAMMER-ILG in their catalytic sub-domain X), which also target SR proteins and affect splicing (13), have also been identified. Both mouse *CLK1* and the *Drosophila* homologue *doa* give rise to transcripts coding for either the full-length catalytically active kinase or a truncated protein lacking the catalytic domain (48, 49). Identical splice forms were also found in human homologues (50). The ratio of these splice products appears to be regulated developmentally in *Drosophila* (48) and in a tissue and cell-specific manner in mammals (49, 50). In addition, in adult mouse tissues or upon stem cell differentiation, larger transcripts, representing partially spliced products that are sequestered in the nucleus and are unavailable for protein translation, were also detected (49, 51).

SRPK1a expressed in mammalian cells was highly active and showed the same substrate specificity as SRPK1. Both isoforms were able to induce alternative splicing of human tau exon 10 in transfected cells. Furthermore, they were similarly expressed in various human tissues, although the level of expression of SRPK1a was significantly lower than that of SRPK1. Despite their high similarity, these two SRPK family members probably exert distinct cellular functions through differential targeting. Using the yeast two-hybrid assay we found that the NH_2-terminal domain of SRPK1a interacts with SAF-B, implying that this isoform is associated with the nuclear matrix. An SAF-B fusion protein can extract SRPK1a but practically no SRPK1 from cell lysates, and antibodies that immunoprecipitate SAF-B can specifically co-precipitate SRPK1a, further confirming the specificity of this interaction. Furthermore, two of the isolated clones in our two-hybrid screen encoded overlapping parts of lamin B. Although this interaction has not yet been ascertained by binding experi-

ments, it is interesting to note that lamins and other proteins containing coiled-coiled α-helices have also been found to interact with S/MAR DNA sequences (52).

An immunofluorescence analysis of the intracellular localization of SRPK1a did not indicate a localization within the nucleus, similar to that observed for SAF-B (32). On the contrary, the cytoplasmic localization of the kinase was predominant. Although the subcellular localization of SRPK1a in interphase cells does not, therefore, support the co-localization of SRPK1a and SAF-B, it has been suggested that the cytoplasmic sequestering of all SRPK family members, mediated by a nonconserved spacer sequence that separates the conserved catalytic kinase domains, may regulate their nuclear functions by controlling their nuclear concentration (22, 23). Thus only a specific fraction of each kinase enters the nucleus, which then may have a distinct subnuclear localization.

Several nuclear matrix proteins were found to interact with members of the splicing machinery and function to the recognition and sequestration of exon sequences during the processing of pre-mRNA (53, 54). Furthermore, a number of recent reports established the existence of macromolecular complexes containing transcriptional and pre-mRNA processing proteins (see Ref. 32 and references therein). SAF-B has been implicated both in transcription (32, 55, 56) and splicing (32, 57). Recently it has been considered as a molecular base where a "transcriptosome complex" could be assembled, in the vicinity of actively transcribed genes (32). Most interestingly, although SAF-B does not contain any alternate RS dipeptides and, therefore, is not a substrate for the SRPKs, it contains a region rich in glutamic acid/arginine repeats (amino acids 585–658), which was part of our yeast two-hybrid clones. The presence of alternating positive and negative residues in this domain is reminiscent of similar dipeptide motifs found in phosphorylated SR domains and might be critical for protein-protein interactions.

SRPK1a may therefore utilize SAF-B as a docking site to phosphorylate splicing factors and other RS domain-containing proteins, that are in close vicinity with the nuclear matrix. Phosphorylation of these proteins may not only be critical to their function but also regulate their interaction with the nuclear matrix. Along these lines we noticed that phosphorylation of five polypeptides, with apparent molecular masses of 29, 36, 50, 80, and 190 kDa, respectively, that co-precipitate with SAF-B, was significantly increased when SRPK1a was overexpressed in 293T cells (data not shown). The identity of those proteins remains unknown at present.

Taken together our data support the concept that mammalian cells have evolved multiple SR protein kinases that are uniquely regulated and targeted. Thus, although various SRPKs show the same substrate specificity, they may perform specialized cellular functions. The identification of additional SRPK family members, together with the delineation of their regulation pathways and the identification and characterization of additional substrates, specifically modified by them, will ultimately contribute to an understanding of the functions of these enzymes.

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