The Subcellular Localization of SF2/ASF Is Regulated by Direct Interaction with SR Protein Kinases (SRPKs)*

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Serine/arginine-rich (SR) proteins play an important role in constitutive and alternative pre-mRNA splicing. The C-terminal arginine-serine domain of these proteins, such as SF2/ASF, mediates protein-protein interactions and is phosphorylated in vivo. Using glutathione S-transferase (GST)-SF2/ASF-affinity chromatography, the SF2/ASF kinase activity was co-purified from HeLa cells with a 95-kDa protein, which was recognized by an anti-SR protein kinase (SRPK) 1 monoclonal antibody. Recombinant SRPK1 and SRPK2 bound to and phosphorylated GST-SF2/ASF in vitro. Phosphopeptide mapping showed that identical sites were phosphorylated in the pull-down kinase reaction with HeLa extracts and by recombinant SRPKs. Epitope-tagged SF2/ASF transiently expressed in COS7 cells co-immunoprecipitated with SRPKs. Deletion analysis mapped the phosphorylation sites to a region containing an (Arg-Ser)8 repeat beginning at residue 204, and far-Western analysis showed that the region is required for binding of SRPKs to SF2/ASF. Further binding studies showed that SRPKs bound unphosphorylated SF2/ASF but did not bind phosphorylated SF2/ASF. Expression of an SRPK2 kinase-inactive mutant caused accumulation of SF2/ASF in the cytoplasm. These results suggest that the formation of complexes between SF2/ASF and SRPKs, which is influenced by the phosphorylation state of SF2/ASF, may have regulatory roles in the assembly and localization of this splicing factor.

Pre-mRNA splicing is an essential process required for the expression of most eukaryotic protein-coding genes. Splicing catalysis occurs in a spliceosome complex (1). Components of the spliceosome include the U1, U2, and U4/U6.U5 small nuclear ribonucleoprotein particles (snRNPs)1 (2) and numerous non-snRNP protein factors (3). The latter include all members of the SR protein family, which play important roles during mammalian spliceosome assembly. All SR proteins have one or two N-terminal RNA-recognition motifs (RRMs) and a C-terminal domain rich in arginine-serine dipeptide repeats (RS domain). The RS domain is involved in protein-protein interactions with related domains of other splicing factors, and these interactions are thought to be important for splice site selection (4, 5).

The SR proteins are phosphorylated at multiple serines located predominantly within the RS domain (6, 7). At least eight members of the SR family contain phosphopeptides that are recognized by the monoclonal antibody mAb104 (8). Analysis of tryptic phosphopeptides derived from SF2/ASF showed that the RS domain of this protein is phosphorylated at multiple sites both in vivo and in vitro (7). Although the physiological role of SR protein phosphorylation is unknown, recent studies suggested that phosphorylation of the RS domain of SF2/ASF enhances the interactions between this domain and the U1–70K polypeptide and that phosphorylation or dephosphorylation cycles may be required for splicing (9, 10).

Several protein kinases can phosphorylate SR proteins, such as SF2/ASF, within its RS domain in vitro. A U1 snRNP-associated kinase was the first kinase activity reported to phosphorylate RS domains although cDNAs encoding this kinase have not been isolated (11). A second kinase, SRPK1, was purified and cloned on the basis of its ability to phosphorylate SC35 or other SR proteins in vitro (6, 12). The Cdk/Sty kinase has an RS domain at its N terminus and was found to interact with several members of the SR protein family in a yeast two-hybrid screen (7). SRPK2, for which we isolated a mouse brain cDNA, also gave the same pattern of serine phosphorylation of SF2/ASF in vitro as SRPK1 (13) and is probably identical to WBP, a cDNA fragment isolated as encoding a WW-domain-binding protein in a two-hybrid screen (14). A human cDNA for SRPK2 was also recently isolated from a fetal brain library (15). In addition to these recently identified kinases, protein kinases C and A can also phosphorylate SF2/ASF in vitro (7), as can the p34cdc2 kinase (32). Moreover, DNA topoisomerase I has been reported to phosphorylate SR proteins although it has no obvious kinase domain (16). Finally, a nuclear envelope-bound kinase activity phosphorylates the RS motif at the N-terminal domain of the lamin B receptor (17).

To identify kinases that bind specifically to SF2/ASF in mammalian cells, we performed pull-down kinase assays using a GST-SF2/ASF fusion protein immobilized on glutathione-Sepharose beads. We found that SRPK1 and SRPK2 interact specifically with the RS domain of SF2/ASF in a manner that depends on the phosphorylation state of SF2/ASF. This interaction appears to modulate the subcellular localization of SF2/ASF.

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‡ The abbreviations used are: snRNP, small nuclear ribonucleoprotein particle; SR protein, serine/arginine-rich protein; SRPK, SR protein-specific kinase; HA, hemagglutinin; GST, glutathione S-transferase; PBS, phosphate-buffered saline; RS domain, domain rich in arginine-serine dipeptide repeat; PMSF, phenylmethylsulfonyl fluoride; IPTG, isopropyl-1-thio-β-D-galactopyranoside; PAGE, polyacrylamide gel electrophoresis; aa, amino acid.
Preparation of HeLa Cell Extracts—HeLa cells were grown in suspension culture. The cells were harvested at the logarithmic growth stage (4–6 × 10^6 cells/ml), suspended in lysis buffer (20 mM HEPES (pH 7.8), 50 mM KCl, 1 mM EDTA, 10 μg/ml leupeptin, 1 μM PMSF, 1% Triton X-100), and incubated on ice for 20 min. The lysates were centrifuged at 12,000 × g for 30 min at 4 °C. Nuclear and S100 extracts were prepared as described (20).

Preparation of Recombinant Proteins—His-tagged SRPK1 and SRPK2 proteins were expressed in Escherichia coli and purified as described (13) using Ni-NTA matrix (QIAGEN). GST-SF2/ASF was expressed in E. coli DH5 and induced with 0.1 mM IPTG for 2 h. The cells were lysed in GST binding buffer (50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 2 mM benzamidine, 2 mM dithiothreitol, 1 mM PMSF, 1% Triton X-100) and passed through a glutathione-Sepharose 4B column (Amersham Pharmacia Biotech). GST-SF2/ASF and its mutant derivatives were eluted with 30 mM glutathione and dialyzed twice against TSE buffer (50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 0.1 mM EDTA, 2 mM benzamidine, 2 mM dithiothreitol, 0.5 mM PMSF, 20% glycerol) for 6 h each at 4 °C.

Full-down Kinase Assay—HeLa cell nuclear extract or recombinant kinases were incubated with GST-SF2/ASF in 1 μl of PBS (137 mM NaCl, 2.7 mM KCl, 4.3 mM NaHPO4, 7H2O, 1.4 mM KH2PO4) for 1 h at 4 °C. Glutathione-Sepharose 4B was added and rotated for 30 min at 4 °C. The beads were washed with cold PBS five times for 5 min each, resuspended in 50 μl of 40 mM HEPES (pH 7.8), 10 mM MgCl2, 2 mM dithiothreitol, and 1 μM of γ-[32P]ATP and incubated for 30 min at 30 °C. The beads were then boiled in SDS-PAGE buffer (1% SDS, 1% mercaptoethanol, 10 mM Tris-HCl (pH 8.0), 20% glycerol, 0.05% bromophenol blue), and the proteins were analyzed on a 10% SDS-polyacrylamide gel.

Phosphopeptide Mapping—Phosphorylation sites of SF2/ASF were analyzed by phosphtopeptide mapping as described before (31) after the pull-down kinase assay. The phosphorylated SF2/ASF bands were excised from SDS-polyacrylamide gels, and the proteins were digested with 10 μl of 50 mM NH4HCO3 (pH 8.4) for 16 h at 37 °C. After digestion, the samples were lyophilized, spotted onto thin layer silica gel plates, and subjected to electrophoresis for 70 min at 1000 V. After digestion, the samples were lyophilized, spotted onto thin layer silica gel plates, and subjected to electrophoresis for 70 min at 1000 V. The gel was then placed onto nitrocellulose membranes. After electrophoresis, the proteins were transferred to a nitrocellulose membrane. The blots were blocked with 5% skim milk for 16 h at room temperature before being incubated in primary antibodies. After incubation, the membranes were washed with PBS and incubated with secondary antibody (AP-conjugated goat anti-rabbit IgG (Zymed Laboratories Inc.) or anti-mouse IgG (Zymed Laboratories Inc.).) The blots were washed twice with PBS and harvested. The membranes were then incubated with secondary antibody (AP-conjugated goat anti-mouse IgG) and visualized with an autoradiogram.

Immunoprecipitations—COS7 cells were cultured at 37 °C in 10-cm dishes in Dulbecco’s modified Eagle’s medium (Nissui Pharmaceutical Co., Ltd.) supplemented with 10% fetal bovine serum and transfected with 20 μg of plasmid DNA in DEAE-dextran. After 48 h, the cells were washed twice with PBS and harvested. The cells were resuspended in 200 μl of lysis buffer (20 mM HEPES (pH 7.8), 150 mM NaCl, 1 mM EDTA, 10 μg/ml leupeptin, 1 μM PMSF, 1% Triton X-100), and incubated on ice for 20 min. The solution was cleared by centrifugation at 15,000 rpm for 30 min at 4 °C. Anti-HA antibody or anti-c-Myc antibody was added to the supernatant, followed by incubation for 30 min at 4 °C. The solution was then incubated with protein G-Sepharose for 30 min at 4 °C. The beads were washed with cold PBS five times for 5 min each. The immunoprecipitates were analyzed by further incubation under phosphorylation conditions, followed by SDS-PAGE and autoradiography, or by Western blotting.

Immunostaining—HeLa cells were grown on coverslips and cultivated in a CO2 incubator for 24 h after transfection. The coverslips were washed twice with PBS. Subsequently, the cells were fixed in 4% paraformaldehyde in PBS for 15 min, at room temperature, permeabilized, and blocked in PBS containing 0.4% Triton X-100, 1% bovine serum albumin, and 5% normal goat serum. For the double immunostaining of the cells expressing HA-SRPKs and myc-SF2, anti-HA polyclonal antibody (MBL), and anti-c-Myc monoclonal antibody (Santa Cruz) were used, followed by incubation with secondary fluorescein isothiocyanate-conjugated goat anti-rabbit IgG (Zymed Laboratories Inc.) and Texas red-labeled goat anti-mouse IgG (Southern Biotechnology Associates, Inc.).

RESULTS

Recombinant human GST-SF2/ASF was expressed in and purified from E. coli. The protein was incubated with HeLa cell extract and recovered by pull-down with glutathione-Sepharose beads. The beads were then used for kinase reactions in the presence of radiolabeled ATP (Fig. 1A). Phosphorylation of the 60-kDa GST-SF2/ASF protein, detected by autoradiography (lane 1), suggested the existence of an SF2/ASF-associated kinase in HeLa cell extracts.

To purify the kinase that binds to and phosphorylates SF2/ASF in HeLa cells, we used affinity chromatography. The extracts were loaded on a GST-SF2/ASF-glutathione-Sepharose column, and bound material was eluted with a salt gradient. Individual fractions were assayed and SF2/ASF kinase activity eluted between 300–500 mM salt, whereas the bulk of bound protein eluted between 100–200 mM salt (Fig. 1B). SDS-PAGE and silver staining analysis of the active fractions revealed a prominent band with a relative molecular mass of 95 kDa (Fig. 1C). The 95-kDa protein reacted with a monoclonal antibody against SRPK1 (Fig. 1D).

The association of SRPK1 in HeLa extracts with GST-SF2/ASF was confirmed by Western blotting of the complexes with anti-SRPK1 antibody (Fig. 2). GST-SF2/ASF or GST were incubated in HeLa cell nuclear (NE, lanes 1 and 3) or cytosolic S100 (lanes 2 and 4) extract and recovered by pull-down with glutathione-Sepharose beads. The complexes were recovered and analyzed by Western blotting with a monoclonal antibody against SRPK1.

The pull-down kinase assays were also carried out using recombinant SRPK1 and SRPK2, instead of the HeLa extract (Fig. 3A). Both SRPK1 and SRPK2 bound stably to and subsequently phosphorylated GST-SF2/ASF under these conditions (lanes 2 and 3), whereas Clk/Sty did not (lane 4).

We compared the phosphorylation sites of SF2/ASF phosphorylated by the GST-SF2/ASF-bound kinase in HeLa extracts (Fig. 3B, panel a), recombinant SRPK1 (Fig. 3B, panel b), or recombinant SRPK2 (Fig. 3B, panel c) using two-dimensional thin layer electrophoresis and chromatography. The resulting patterns are indistinguishable. These results suggest that the SRPKs are the major kinases capable of associating with GST-SF2/ASF in HeLa cell extracts.

To determine whether SRPKs interact with SF2/ASF in vivo, we carried out immunoprecipitation experiments. HA-tagged SRPK1 or SRPK2 and myc-tagged SF2/ASF expression plasmids were transiently transfected into COS7 cells, and the cells were harvested and lysed at 48 h post-transfection. Immunoprecipitations were carried out with either anti-c-Myc (Fig. 4A) or anti-HA monoclonal antibodies (Fig. 4B), and the immunoprecipitates were then incubated in kinase buffer in the presence of radiolabeled ATP. Phosphorylated SF2/ASF was detected only when both SRPK and SF2/ASF expression plasmids were co-transfected (Fig. 4A, lanes 2 and 3, Fig. 4B, lanes 2 and 3), although the endogenous kinase gave a faint band (Fig. 4A, lane 1). These results indicate that SF2/ASF and the SRPKs can form stable complexes in vivo. These results were con-
A monoclonal antibody against SRPK1. Column fractions as in Fig. 1. The molecular masses of standards are indicated on the left axis. Closed circles indicate total protein concentration, as indicated on the right y-axis. Open circles each fraction was determined with a DC protein assay kit (Bio-Rad). (13). Fractions of 1 ml were collected, and the protein concentration of each fraction was determined with a DC protein assay kit (Bio-Rad). (13). Fractions of 1 ml were collected, and the protein concentration of each fraction was determined with a DC protein assay kit (Bio-Rad). (13). Fractions of 1 ml were collected, and the protein concentration of each fraction was determined with a DC protein assay kit (Bio-Rad).

B Purification of SF2/ASF-bound kinases from HeLa cell extracts. A, GST-SF2/ASF was incubated in HeLa cell nuclear extract, recovered by pull-down with glutathione-Sepharose beads, and incubated under in vitro phosphorylation conditions in the presence of [γ32P]ATP. Phosphorylation was detected by SDS-PAGE and autoradiography. Lane 1, phosphorylation of GST-SF2/ASF; lane 2, negative control with unfused GST. B, elution profile of proteins bound to immobilized GST-SF2/ASF. HeLa cell extract was applied to a GST-SF2/ASF-glutathione-Sepharose column. A linear gradient from 50 to 500 mM NaCl was applied from fractions 16 to 25. 25-μl aliquots were recovered by pull-down with glutathione-Sepharose beads. The complexes were recovered and analyzed by Western blotting with a monoclonal antibody against SRPK1. The mobility of SRPK1 is indicated on the right.

C Western blotting of GST-SF2/ASF-bound SRPK1 of HeLa cells. GST or GST-SF2/ASF were incubated in HeLa cell nuclear (NE, lanes 1 and 3) or cytosolic S100 (lanes 2 and 4) extracts and recovered by pull-down with glutathione-Sepharose beads. The complexes were recovered and analyzed by Western blotting with a monoclonal antibody against SRPK1. The mobility of SRPK1 is indicated on the right.

D Confirmation by immunoprecipitation/Western analysis (Fig. 4C). Complexes between SF2/ASF and SRPKs were immunoprecipitated with anti-HA polyclonal antibodies and separated by SDS-PAGE. The proteins were then transferred to nitrocellulose, and epitope-tagged SF2/ASF was detected with anti-c-Myc monoclonal antibody. When myc-tagged SF2/ASF was co-transfected with HA-tagged SRPK1 or SRPK2, the 35-kDa myc-tagged SF2/ASF polypeptide was detected in the anti-HA immunoprecipitate (Fig. 4C, lanes 2 and 3), again demonstrating a stable interaction between SF2/ASF and the SRPKs.

Because the RS domain of SR proteins is thought to mediate protein-protein interactions (4, 5), we constructed several deletion mutants of the RS domain of SF2/ASF to determine what regions are required for stable interaction with the SRPKs (Fig. 5A). The deleted proteins, ΔRS (aa 1–197), Δ204 (aa 1–203), Δ227 (aa 1–226), Δ234 (aa 1–233), and Δ238 (aa 1–237), as well as the wild-type parent protein (aa 1–248) were prepared as GST-fusion proteins in E. coli (Fig. 5B, top). The ability of these proteins to interact with SRPKs was examined by a far-Western assay. The recombinant proteins were separated by SDS-PAGE and blotted onto nitrocellulose. The filters were incubated with recombinant HA-SRPK1, and the bound kinase was detected using anti-SRPK1 antibody (Fig. 5B, middle). SRPK1 bound to Δ227, Δ234, and Δ238 but not to ΔRS or Δ204. The latter two mutants lack a segment of eight consecutive RS dipeptide repeats. In agreement with these results, Δ227, Δ234, and Δ238 were phosphorylated by SRPK1 in vitro, whereas neither ΔRS nor Δ204 proteins could be phosphorylated (Fig. 5B, bottom). Identical results were obtained with SRPK2 (data not shown). Together, these results show that the SRPK phosphorylation sites in SF2/ASF are located within a region containing the eight RS repeats (aa 204–226) and that the same site is required for stable interaction between these kinases and SF2/ASF.

Next, we examined the effect of SF2/ASF phosphorylation on protein-protein interactions between SF2/ASF and SRPK1. The phosphorylation state of SF2/ASF was checked by the mobility shift on SDS-PAGE (Fig. 6, left panel). The interaction of HA-tagged SRPK1 with either phosphorylated (ATP+) or unphosphorylated (ATP-) SF2/ASF was examined by the far-Western assay (Fig. 6, right panel), and the interaction efficiencies were quantitated by the relative intensity of the staining for HA-SRPK1. The SRPK1 bound to unphosphorylated SF2/ASF was 5 times more than that bound to phosphorylated SF2/ASF, indicating that the interaction between these proteins depends on the phosphorylation state of SF2/ASF.

As addition of SRPK1 kinase to permeabilized cells or over-
Expression of Clk/Sty kinase results in a diffuse distribution of SC35, it has been suggested that hyperphosphorylation of the RS domains may control the subcellular distributions of SR proteins (6, 7, 18). We likewise observed that expression of SRPK2 induced changes in the subcellular localization of transiently expressed SF2/ASF (Fig. 7, g and h) and endogenous SC35 (13).

To examine the effect of direct interaction of SF2/ASF with SRPKs on the subcellular localization of SF2/ASF, we analyzed the distribution of SF2/ASF in cells expressing a catalytically inactive kinase mutant, SRPK2K108R, and observed that SF2/ASF accumulated in the cytoplasm of cells expressing the inactive SRPK2 (Fig. 7, i and j). In contrast, a mutant that lacks the presumed SF2/ASF-interaction domain, had no effect on the distribution of SF2/ASF (data not shown).

**DISCUSSION**

SR proteins are essential splicing factors that promote splice-site recognition at an early stage of spliceosome assembly and also influence the selection of alternative splice sites (19, 20, 21, 22). The phosphorylation state of SR proteins appears to influence their activities in general, and alternative splicing, as well as their subnuclear localization and nuclear-
components are concentrated, and they are thought to represent sites of storage or assembly for splicing factors (25). SRPK1 was originally identified as a kinase of SC35 in extracts from HeLa cells (6, 12). Addition of purified SRPK1 to permeabilized cells or overexpression of SRPK1 or Clk/Sty in transfected cells results in an apparent disassembly of the nuclear speckles (6, 7). These results suggest that phosphorylation, or hyperphosphorylation, causes release of these factors from the speckles, or perhaps that the integrity of these structures is compromised.

Information about kinases that can phosphorylate SR proteins has recently been obtained. Although the kinase domains of SRPK1 and Clk/Sty are approximately 30% identical, including key amino acids that are expected to be involved in substrate specificity (6), SRPK1 has a more restricted substrate specificity in vitro (26). Clk/Sty phosphorylates the basic proteins histone H1 and myelin basic protein, albeit to a lower specific activity than it phosphorylates SF2/ASF. It does not phosphorylate more acidic substrates, such as β-casein, enolase, or an N-terminal region of c-Jun (26). SRPK1 was calculated to be 150-fold more active with SF2/ASF than Clk/Sty with SF2/ASF as the substrate (26). The \( K_s \) values for SF2/ASF were similar (0.28 \( \mu M \) for SRPK1, 0.40 \( \mu M \) for Clk/Sty), indicating that SRPK1 turns over SF2/ASF much faster than Clk/Sty does. Our present findings are consistent with these published data. We found that SRPK1 and SRPK2 interact more strongly with SF2/ASF than Clk/Sty does but that this interaction is weakened upon phosphorylation, allowing product release.

Clk/Sty has an RS domain at its N-terminus, through which it is known to interact with five RNA-binding proteins, including three SR proteins, in a two-hybrid screen (7). Our pull-down kinase assay failed to detect an interaction between SF2/ASF and Clk/Sty. The discrepancy may be attributable to the higher sensitivity of the two-hybrid screen for what may be weak or transient interactions, or perhaps the phosphorylation states of the SF2/ASF and Clk/Sty RS domains are different in vitro and in yeast, with the latter resulting in more stable interactions. Alternatively, the interaction of the Clk/Sty RS domain with SF2/ASF may be weak but long lived compared with the interaction between the SRPKs and SF2/ASF, resulting in the slower release of SF2/ASF from Clk/Sty.

SRPK1 was reported to be closely related in sequence to a hypothetical Caenorhabditis elegans kinase (CEHK) and to the fission yeast kinase Dsk1 (6, 27, 28). We recently cloned a mouse brain cDNA encoding a novel SR protein-specific kinase, SRPK2 (13). The amino acid sequence of SRPK2 is 58% identical to that of human SRPK1 and 32% similar to that of yeast Dsk1. Immunolocalization experiments showed that both

Fig. 4. Effect of SF2/ASF phosphorylation state on interaction with SRPK1. Left, Coomassie Brilliant Blue (CBB) staining; right, GST-SF2 was incubated with SRPK1 in the presence (+) or absence (−) of ATP. Phosphorylated (+) or unphosphorylated (−) SF2/ASF were resolved by SDS-PAGE and transferred to a nitrocellulose membrane. The membrane was incubated with SRPK1 and probed with anti-SRPK1 antibody as in Fig. 5B.
Localization of endogenous SF2/ASF (HeLa cells were co-transfected with plasmids expressing myc-tagged secondary antibody (clonal antibody followed by fluorescein isothiocyanate-conjugated sec-
an anti-c-Myc monoclonal antibody followed by Texas red-conjugated transfected HeLa cells using an anti-SF2/ASF monoclonal antibody or myc-SF2/ASF only (SRPK1 wild type (SRPK2K108R may reflect the differential roles of Clk/Sty and their subcellular localization. Thus, the activation state of SR proteins may be a key determinant of the subcellular distribution of SF2/ASF. In support of this notion, expression of a deletion mutant of SRPK1, which lacks the presumed SF2/ASF-interaction domain, had no effect on the localization of SF2/ASF (data not shown).

SRPK1 activity was shown to be highest in metaphase cells, suggesting that SRPK1 expression or activity is subject to cell cycle control (6). The fact that recombinant SRPK1 expressed in E. coli binds to and phosphorylates SF2/ASF suggests the existence of a cell-cycle regulated kinase inhibitor or inhibitory modification of the kinase in mammalian cells. An inactive form of SRPKs would still be expected to form a stable complex with SF2/ASF in the cytoplasm. In this study, we obtained approximately 6 mg of SRPK1 from 0.9 g of HeLa cells by GST-SF2/ASF affinity chromatography. The amount of endogenous SRPKs seems to be relatively high in comparison with the endogenous kinase activity measured by the pull-down kinase assay, suggesting that the majority of SRPKs may be free from SF2/ASF in living cells. Considering that SRPK1 is inactive through G1/S phase (6), our results suggest the existence of a potential regulator that works as a pseudosubstrate, although other regulatory mechanisms cannot be eliminated. Thus, the activation state of SRPKs may determine the assembly of SR proteins into kinase-substrate complexes, as well as their subcellular localization.

SF2/ASF Localization Is Regulated by Interaction with SRPK

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FIG. 7. Effect of SRPKs expression on SF2/ASF distribution. HeLa cells were co-transfected with plasmids expressing myc-tagged SF2/ASF and HA-tagged SRPKs and then fixed 24 h after transfection. Localization of endogenous SF2/ASF (a and d) and myc-SF2/ASF (b, f, h, and j) were visualized by indirect immunofluorescence staining of transfected HeLa cells using an anti-SF2/ASF monoclonal antibody or an anti-c-Myc monoclonal antibody followed by Texas red-conjugated secondary antibody. HA-SRPks were detected using an anti-HA polyclonal antibody followed by fluorescein isothiocyanate-conjugated secondary antibody (c, e, g, and i). Bar, 10 µm. Endogenous SF2/ASF (a), myc-SF2/ASF only (b), SRPK1 wild type only (c and d), myc-SF2/ASF with SRPK1 wild type (e and f), SRPK2 wild type (g and h), and SRPK2K108R (i and j), respectively.

SRPK1 and SRPK2 are primarily localized in the cytoplasm, despite having putative nuclear localization signals (13). In contrast, SF2/ASF accumulates in the nucleus, concentrating in the speckle domains (29). The RS domain of SF2/ASF is a nuclear targeting signal, although it is not sufficient to direct accumulation in the speckle domains. The RS domain of SF2/ASF is also involved in shuttling of the protein between the nucleus and the cytoplasm, and co-expression of Clk/Sty, presumably resulting in hyperphosphorylation of SF2/ASF, interferes with shuttling, resulting in accumulation of SF2/ASF in the cytoplasm (30). Expression of a catalytically inactive kinase mutant, Clk/StyK190R had a much more limited effect on the distribution of SR proteins, suggesting that the phosphorylation state of SR proteins strongly influences their cellular distribution. The present study, however, shows that SF2/ASF accumulated in the cytoplasm even in cells expressing the catalytically inactive kinase SRPK2K108R. The discrepancy in the effects of kinase-inactive mutants Clk/StyK190R and SRPK2K108R may reflect the differential roles of Clk/Sty and SRPK in vivo. The stable interactions between SF2/ASF and SRPKs may be a key determinant of the subcellular distribution of SF2/ASF. In support of this notion, expression of a deletion mutant of SRPK1, which lacks the presumed SF2/ASF-interaction domain, had no effect on the localization of SF2/ASF (data not shown).
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