Phosphorylation-dependent and independent Nuclear Import of RS Domain-containing Splicing Factors and Regulators*

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SR proteins and related RS domain-containing polypeptides are an important class of splicing regulators in higher eukaryotic cells. The RS domain facilitates nuclear import of SR proteins and mediates protein-protein interactions during spliceosome assembly; both functions appear to subject to regulation by phosphorylation. Previous studies have identified two nuclear import receptors for SR proteins, transportin-SR1 and transportin-SR2. Here we show that transportin-SR1 and transportin-SR2 are the alternatively spliced products of the same gene and that transportin-SR2 is the predominant transcript in most cells and tissues examined. While both receptors import typical SR proteins in a phosphorylation-dependent manner, they differentially import the RS domain-containing splicing regulators hTra2α and hTra2β in different phosphorylation states. We suggest that differential regulation of nuclear import may serve as a mechanism for homeostasis of RS domain-containing splicing factors and regulators in the nucleus and for selective cellular responses to signaling.

Small nuclear ribonucleoprotein particles and a large number of protein factors are required for the assembly of spliceosomes, where pre-mRNA is processed into mature mRNA (1, 2). SR proteins are a family of non-small nuclear ribonucleoprotein particles splicing factors that are important for both constitutive and regulated splicing (3). Typical SR proteins contain one or two RNA recognition motifs, which are responsible for their sequence-specific RNA binding activities, and an RS domain enriched with arginine and serine repeats in the C terminus (4). Many biochemically and genetically identified splicing regulators, such as Tra and Tra2 in the Drosophila sex determination pathway, are related to SR proteins by the presence of an RS domain (3, 4). Interestingly, two Tra2 homologues, hTra2α and hTra2β, appear to function as general sequence-specific splicing activators in mammalian cells (5).

All SR proteins are extensively modified by phosphorylation, which has been shown to be critical for RS domain-mediated protein-protein interactions important for spliceosome assembly (6–8). Both hypo- and hyperphosphorylation, however, appear to be inhibitory to the function of SR proteins in splicing (9), but the precise mechanism for why a hyperphosphorylated SR is inhibitory remains to be understood, especially in light of a recent observation that RS repeats in the RS domain of the SR protein ASF/SF2 can be substituted by RE or RD to mimic the hyperphosphorylation state in splicing (10). This may be related to the question whether modulation of SR protein function by phosphorylation is due to changes in overall charge distribution brought by phosphorylation in the RS domain, or more specifically, to the modification at some critical sites, or perhaps, to both.

SR proteins are distributed in a speckled pattern in interphase nuclei and become dispersed throughout the cell during mitosis as a result of hyperphosphorylation (11). More recently, hyperphosphorylated SR proteins were found to be restricted from the nucleus before zygotic activation of gene expression in nematodes (12). These observations suggest that phosphorylation may represent an important mechanism to regulate the activity and localization of SR proteins during the cell cycle as well as in development.

Although SR proteins are localized in the nucleus at steady state, a subset of SR proteins is known to shuttle between the nucleus and the cytoplasm (13). It is thought that SR protein shuttling may reflect a role in mRNA transport (14) or a way to regulate their function by localization (15). A functional link between nuclear import and phosphorylation regulation of SR proteins was first established in budding yeast where defects in a conserved SR protein kinase resulted in impaired nuclear import of an SR-like RNA binding protein Npl3p (16, 17). Npl3p is imported by an importin-β-related Ran-binding protein Mtr10p (18, 19). Likewise, SR proteins can be imported by an Mtr10p homologue identified as transportin-SR (TRN-SR1) in mammalian cells (20). More recently, a second SR protein import receptor known as transportin-SR2 (TRN-SR2) and its Drosophila ortholog (dTRN-SR2) were cloned and characterized (21, 22). Although the requirement for phosphorylation in TRN-SR1-mediated nuclear import of SR proteins remains to be investigated, both Mtr10p-mediated nuclear import of Npl3p in yeast (16) and TRN-SR2-mediated nuclear import of SR proteins in mammalian cells (23) have been shown to be phosphorylation-dependent.

Here we investigated the relationship between TRN-SR1 and TRN-SR2 and found that they are actually alternatively spliced products of the same gene in mammalian cells. We also show that both TRN-SR1 and TRN-SR2 import typical SR proteins in a phosphorylation-dependent manner. Interestingly, TRN-SR1 and TRN-SR2 demonstrate distinct requirements for phosphorylation in mediating nuclear import of RS domain-containing splicing regulators hTra2α and hTra2β.

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1 The abbreviations used are: TRN, transportin; RT, reverse transcriptase; GST, glutathione S-transferase; NLS, nuclear localization signal.
The results have important implications for how SR proteins might be coordinately regulated in vivo.

**EXPERIMENTAL PROCEDURES**

**Construction of a TRN-SR1/SR2 Minigene for In Vitro Splicing—**PCR amplification of the genomic sequences surrounding TRN-SR1/SR2 exons 10 and 11, as indicated in Fig. 1B, was carried out using two sets of primers (5′-gggaATTCTAACCCTTGAAAGGGAACC-3′ and 5′-ggggctATGTTTCCTCCCTGTTG-3′; 5′-gggtacGCTTCTCTGCTTTT-3′ and 5′-gctagaAGTTGGCGAGGAGGAGGA-3′; EcoRI, KpnI, or XhoI sites for cloning are underlined, and capital letters are those present in the TRN-SR1/SR2 genomic sequences). Digestion PCR fragments were cloned into the EcoRI and XhoI sites in pSP72 (Promega). In vitro transcription by SP6 RNA polymerase and in vitro splicing using HEla nuclear extracts were performed under standard conditions.

**RT-PCR Analysis—**Total RNA was extracted from different human cell lines using TRIzol (Invitrogen). A panel of human tissue total RNA was purchased from Clontech. For RT-PCR, 5 μg of total RNA was reverse-transcribed in a 15-μl reaction using the Moloney murine leukemia virus reverse transcriptase (Promega), and 1 μl was used for PCR. The forward and reverse primers for the human TRN-SR1/SR2 gene are 5′-CCGATGAGGGTATCAGACCT-3′ and 5′-GAGGGCGAGGAGGAGAGGAGGA-3′; EcoRI, KpnI, or XhoI sites for cloning are underlined. The PCR reactions were carried out using AmpliTaq Gold (Applied Biosystems) for 10 min at 95 °C (activation and denaturation) followed by 35 cycles at 94 °C for 30 s, 60 °C for 30 s, and 72 °C for 30 s. The PCR products were analyzed in 2% agarose gel. Expected PCR products from cloned TRN-SR1 (312 nucleotides) and TRN-SR2 (210 nucleotides) plasmids were amplified with equal efficiencies, which served as controls for RT-PCR analysis of total RNA from human tissues and cell lines. No band was detected from isolated total RNA when RT was omitted.

**Preparation of Recombinant Proteins for In Vitro Binding and Nuclear Import Assay—**Bacterially produced GST-ASF/SF2, GST-hTra2α, and GST-hTra2β have been described previously (8). Bacterially produced His-S-TRN-SR1, His-S-TRN-SR2, His-GFP-ASF/SF2, His-GFP-hTra2α, and His-GFP-hTra2β proteins were purified on nickel columns.

**In Vitro Modifications, Binding, and Nuclear Import—**Phosphorylation of GST-ASF/SF2, GST-hTra2α, and GST-hTra2β by SRPK1 and subsequent in vitro bindings were performed as described previously (8). Briefly, bacterially expressed GST fusion proteins were phosphorylated for 6 h, which resulted in a dramatic mobility shift in SDS gel in all cases. It was estimated by quantifying incorporated radioactive ATP that 8 to 9 phosphates were added to GST-ASF/SF2, 15 to 16 to GST-hTra2α, and 17 to 18 to GST-hTra2β. After desalting, 1 μg of GST fusion protein was bound to glutathione-Sepharose (Amersham Biosciences) and the mixture was incubated with 1 μl of recombinant His-S-TRN-SR1 or His-S-TRN-SR2. After binding and extensive washing, bound His-S-tagged receptor was detected by Western blotting using a His antibody (Clontech) or the S-tag HRP LumiBlot kit (Novagen).

The in vitro import assay was described as performed previously (26). Briefly, HeLa cells cultured on coverslips with 50% density (about 5 × 10^5 cells) were permeabilized with digitonin (Calbiochem), and before each transport reaction, 90–95% permeabilization was confirmed by staining with trypan blue. Permeabilized cells were incubated with a 20-μl import mixture at 30 °C for 30 min. Import reactions with cytosol contained 5 μl of HeLa cytosol, ATP regeneration system (1 mM ATP, 5 mM creatine phosphate, 10 units/ml creatine phosphate kinase), and 0.5 μg of GFP-tagged transport cargo. Import reactions with purified components contained the ATP regeneration system, 150 μg of Ran, 250 μg of NTF2, 0.5 μg of GFP-tagged cargo that was phosphorylated or mock-phosphorylated by SRPK1, and 0.5 μg of His-S-TRN-SR2 or His-S-TRN-SR2. After import, cells were washed in transport buffer, fixed in 3.7% formaldehyde, and analyzed by fluorescence microscopy. Control for positive import was an identical reaction incubated on ice.

**RESULTS**

**TRN-SR1 and TRN-SR2 Are Differentially Spliced Products—**Sequence comparison reveals that TRN-SR1 and TRN-SR2 are identical except for two regions, one in the middle and the other at the C terminus. Alignment of their cDNA sequences indicates that TRN-SR1 contains an in-frame insertion of 102 nucleotides (34 amino acids) in the middle and a deletion of a nucleotide (G) near the C terminus, the latter of
which appears to result from a sequencing error, which has been confirmed by re-sequencing of the cloned TRN-SR1 and the corresponding genomic regions in both mice and humans (data not shown). Thus, TRN-SR1 and TRN-SR2 have the same C termini. The overall sequence identity at the nucleotide level between TRN-SR1 and TRN-SR2 suggests that they may result from alternative splicing. Indeed, sequence alignment between cDNA and genomic sequences in both mice and humans reveals that the insertion in TRN-SR1 is due to the use of a proximal 3' splice site relative to the 3' splice site for TRN-SR2 in exon 11 (Fig. 1).

Sequence comparison with consensus splicing sites indicates that the 3' splice site for TRN-SR1 contains a poor branchpoint sequence and a strong polyadenylation tract, whereas the 3' splice site for TRN-SR2 consists of a strong branchpoint sequence and a strong polyadenylation tract (Fig. 1B). In addition, we note another potential cryptic 3' splice site further upstream the two 3' splice sites (see a long polyadenylation tract followed by a bold ag in Fig. 1B). Such a sequence arrangement implies that multiple 3' splice sites in the front of exon 11 may compete with one another in conjunction with other potential regulatory elements to give rise alternatively spliced TRN-SR1 and TRN-SR2. To determine whether these 3' splice sites are indeed competent, we isolated by PCR two TRN-SR1/SR2 genomic fragments surrounding exon 10 and exon 11 and constructed a minigene for in vitro splicing. As shown in Fig. 2A, the TRN-SR1/SR2 minigene transcript was inefficiently spliced in HeLa nuclear extracts relative to the globin (Hb) control. Nevertheless, the 5' splice donor was spliced to all three competing 3' splice sites, but with descending efficiency. These observations indicate that all potential splice sites are competent, at least in vitro, but their selection in vivo may subject to regulation.

TRN-SR2 Is Predominantly Expressed in Most Cell Types—TRN-SR1 was originally isolated from a HeLa cell library (20). However, this isoform is underrepresented in cDNA and EST databases, indicating that TRN-SR1 may be a rare isoform. RT-PCR analysis of a panel of human tissues and cell lines

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*N. Kataoka, personal communication.*
showed that TRN-SR1 is undetectable and TRN-SR2 is the predominant isoform in most cell types (Fig. 2B). Thus, the cryptic 3’ splice site and that for TRN-SR1 appear to be effectively suppressed in vivo in most cases, even though the 3’ splice site for TRN-SR1 appears to be stronger than that for TRN-SR2 in in vitro splicing of the minigene (Fig. 2A). It remains to be determined whether TRN-SR1 is only expressed in some special cell types or induced in response to certain signaling pathways (see “Discussion”).

Both TRN-SR1 and TRN-SR2 Bind and Import SR Proteins in a Phosphorylation-dependent Manner—A previous study has shown that TRN-SR1 is capable of binding and importing GST fused to RS domains from two prototypical SR proteins, ASF/SF2 and SC35 (20). In this study, only unphosphorylated RS domain fusion proteins were tested and potential impact of phosphorylation on TRN-SR1-mediated nuclear import was not addressed. Subsequently, Tarn and colleagues (21, 23) reported cloning and characterization of TRN-SR2 (21), which appears to bind and import phosphorylated ASF/SF2 in a strictly phosphorylation-dependent fashion. To determine whether the two isoforms indeed exhibit distinct requirements for phosphorylation in binding and importing SR proteins, we carried out the in vitro binding assay using GST-ASF/SF2 and His- or S-tagged TRN-SR1 and TRN-SR2, all produced and purified from bacteria, under identical conditions. GST-ASF/SF2 was either mock-phosphorylated or phosphorylated by recombinant SRPK1, a kinase specific for SR proteins (11). As shown in Fig. 3A, both TRN-SR1 and TRN-SR2 bound GST-ASF/SF2 in a phosphorylation-dependent manner. Similar phosphorylation-dependent binding of GST-ASF-RS and GST-SC35-RS to TRN-SR1 and TRN-SR2 was also obtained (data not shown). Thus, both versions of SR protein import receptors appear to behave in a similar way in their interactions with typical SR proteins. A low level of specific interaction with unphosphorylated GST-ASF/SF2 by TRN-SR1 or 2 was detectable, which may explain the reported interaction between TRN-SR1 and unphosphorylated RS domain fusion proteins, especially when a large amount of recombinant RS domain fusion proteins was used in initial binding and import reactions (20).

To verify phosphorylation-dependent nuclear import of SR proteins by both TRN-SR1 and TRN-SR2, we performed in vitro nuclear import assays that utilize digitonin-permeabilized HeLa cells (26). As a control, we tested the import of a fluorescein isothiocyanate-labeled substrate carrying a classical NLS from the SV40 T antigen. This substrate was actively imported this SR protein in a phosphorylation-dependent manner (Fig. 3B). A GFP-ASF/SF2 fusion protein was similarly imported in the presence of cytosol (source of import components) (Fig. 3B). A GFP-ASF/SF2 fusion protein was similarly imported as demonstrated previously (20, 23). Recombinant TRN-SR1 and TRN-SR2 were then tested in this in vitro system for their ability to import mock- and SRPK1-phosphorylated GFP-ASF/SF2. The results showed that both receptors actively imported this SR protein in a phosphorylation-dependent manner (Fig. 3C). Similar phosphorylation-dependent binding and import were also obtained with GFP-SC35-RS domain fusion protein (data not shown). We therefore conclude that TRN-SR1 and TRN-SR2 function similarly in binding and
importing typical SR proteins tested.

Phosphorylation Is Differentially Required for Nuclear Import of hTra2α and hTra2β—We then extended the analysis to additional RS domain-containing splicing factors. We were particularly interested in testing hTra2α and hTra2β, because they can be considered either as typical SR proteins based on their general splicing enhancer function in mammalian cells (5) or as RS domain-containing splicing regulators due to their sequence similarity to the splicing regulator Tra2 in the Drosophila sex determination pathway and their functions in regulated splicing in the neuron (27). In contrast to phosphorylation-dependent interactions with ASF/SF2, both TRN-SR1 and TRN-SR2 appeared to bind hTra2α specifically, but phosphorylation did not seem to influence the binding (Fig. 4A). Titration of TRN-RS1 and TRN-SR2 indicated that the interaction of these two receptors with both unphosphorylated and phosphorylated hTra2α was unlikely due to saturating amounts of the receptors used in the binding reactions (Fig. 4A). In contrast, while TRN-SR1 exhibited efficient interaction with hTra2β, regardless of its phosphorylation state, TRN-SR2 bound hTra2β in a manner that was highly dependent on phosphorylation (Fig. 4A). As expected, the efficiency of nuclear import paralleled the binding profile. Both TRN-SR1 and TRN-SR2 were capable of importing unphosphorylated and phosphorylated hTra2α with similar efficiencies (Fig. 4B). In contrast, TRN-SR1 was able to import both unphosphorylated and phosphorylated hTra2β, but TRN-SR2 could only import phosphorylated hTra2β (Fig. 4C). These data indicate that phosphorylation may not be universally required for nuclear import of all RS domain-containing splicing factors and regulators. This would allow this class of splicing factors enter the nucleus via phosphorylation-dependent and/or phosphorylation-independent pathways, and thus, the process may be differentially regulated by phosphorylation in different tissues or cell types (see “Discussion”). In certain cases, it would not be surprising that phosphorylation may actually prevent nuclear entrance of certain splicing suppressors in interphase cells, such as SRp30 described recently (28).

DISCUSSION

The current study has clarified the structural relationship between TRN-SR1 and TRN-SR2 and extended previous work on the function of these two closely related nuclear import receptors for RS domain-containing splicing factors and regulators. Based on our expression survey by RT-PCR (Fig. 2B), it is clear that, in most human tissues and cell lines examined, TRN-SR1 is rare and TRN-SR2 is a predominant isoform. This expression profile is in contrast to that from in vitro splicing of the TRN-SR1/TRN-SR2 minigene transcript, which was designed to contain all identifiable exonic and intronic sequence elements including a portion of intronic sequence downstream exon 11 for potential exon definition effect (29). Further experiments will thus be required to test additional sequences in intron 10 to understand why the upstream cryptic splice site and the 3′ splice site for TRN-SR1 are used in in vitro splicing, but effectively suppressed in most cell types. Our current results also leave open the possibility that TRN-SR1 might be expressed in some special tissue and cell types or might be induced by certain signaling or developmental cues. If the expression of TRN-SR1 can be detected in future studies, it will serve as an interesting model to investigate how such tight control of splice site selection is achieved in mammalian cells.

Although previous studies have documented a role for TRN-SR1 and TRN-SR2 in mediating nuclear import of SR proteins, our current study reveals their functional similarities as well as differences in binding and importing RS domain-containing splicing factors and regulators. In particular, hTra2α seems to be imported by both receptors in a phosphorylation-independent pathway whereas hTra2β appears to be differentially imported, depending on its phosphorylation state. Interestingly, like their Drosophila homologue, both hTra2α and hTra2β appear to harbor two RS domains, one at the N terminus and the other at the C terminus. Previous mutagenesis studies have established the presence of multiple NLS sequences in RS domain-containing splicing factors and regulators (30, 31), but it is unclear whether those seemingly redundant NLS sequences in native proteins function in a parallel or synergistic fashion. In addition, sequences outside the RS domain of ASF/SF2 may also carry an independent nuclear targeting signal (10). In the case of hTra2α and -β, however, preliminary binding studies using deletion mutants indicate that their import by TRN-SR1 and TRN-SR2 appears to be mediated by multiple nuclear targeting signals in the RS domains, not through their RNA recognition motifs.3 Fine mapping is in progress to dissect NLS in each RS domain and determine which NLS-mediated nuclear import depends on phosphorylation and which does not.

A detailed characterization of how RS domains interact with TRN-SR1 and TRN-SR2 also remains to be done, which is crucial for understanding the molecular basis for phosphorylation-dependent and -independent interactions. Structural and functional studies of the prototypical receptor importin-β show that it can interact with the IBB motif in the adaptor protein importin-α (32) as well as directly with a variety of NLS sequences in small nuclear ribonucleoprotein particles (33), transcription factors (34, 35), cell cycle regulators (36), and arginine-rich human immunodeficiency virus regulatory proteins (37, 38). Thus, a variety of different types of molecular interactions may take place in the cargo binding pocket of importin-β. A similar situation may also hold true for the interaction between TRN-SR1 or TRN-SR2 and different RS domains, but

3 C. Y. Yun, A. L. Velazquez-Dones, and X.-D. Fu, unpublished observation.
this will require detailed structural and functional studies in the future.

From the point of view of splicing regulation, nuclear import of different RS domain-containing proteins in more than one pathway may be important for a network control of gene expression at the splicing level. For splicing factors imported in the phosphorylation-dependent pathway, the level of such factors in the nucleus may subject to regulation by the net effects of kinase and phosphatase activities in the cell, as illustrated in Fig. 5A. Unimported factors may be directed to a degradation pathway in the cytoplasm, which might be a mechanism for maintaining homeostasis of some splicing factors and regulators in specific cell types. In considering the possibility that the TRN-SR1/SR2 gene might be regulated by alternative splicing in some special cell types by RS domain-containing proteins, one may speculate that import of one splicing regulator may alter the ratio of TRN-SR1 and TRN-SR2 to affect import of other RS domain-containing splicing factors, thereby creating a chain of regulatory events (Fig. 5B). For example, changes in the balance between SR protein kinase and phosphatase activities might differentially affect nuclear import and consequently alter the effective concentration of RS domain-containing splicing regulators in the nucleus. As a result, a switch from TRN-SR2 to TRN-SR1 might be induced under certain conditions. The production of TRN-SR1 would then facilitate nuclear import of some RS domain-containing proteins, such as hTraf2β, via the phosphorylation-independent pathway. This potential mechanism would allow cells to selectively import a subset of RS domain-containing splicing factors and regulators in response to signaling. This hypothesis could be tested in the future if cells capable of expressing TRN-SR1 can be identified.

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