A Conserved *Drosophila* Transportin-Serine/Arginine-rich (SR) Protein Permits Nuclear Import of *Drosophila* SR Protein Splicing Factors and Their Antagonist Repressor Splicing Factor 1

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Members of the highly conserved serine/arginine-rich (SR) protein family are nuclear factors involved in splicing of metazoan mRNA precursors. In mammals, two nuclear import receptors, transportin (TRN)-SR1 and TRN-SR2, are responsible for targeting SR proteins to the nucleus. Distinctive features in the nuclear localization signal between *Drosophila* and mammalian SR proteins prompted us to examine the mechanism by which *Drosophila* SR proteins and their antagonist repressor splicing factor 1 (RSF1) are imported into nucleus. Herein, we report the identification and characterization of a *Drosophila* importin β -family protein (dTRN-SR), homologous to TRN-SR2, that specifically interacts with both SR proteins and RSF1. dTRN-SR has a broad localization in the cytoplasm and the nucleus, whereas an N-terminal deletion mutant colocalizes with SR proteins in nuclear speckles. Far Western experiments established that the RS domain of SR proteins and the GRS domain of RSF1 are required for the direct interaction with dTRN-SR, an interaction that can be modulated by phosphorylation. Using the yeast model system in which nuclear import of *Drosophila* SR proteins and RSF1 is impaired, we demonstrate that complementation with dTRN-SR is sufficient to target these proteins to the nucleus. Together, the results imply that the mechanism by which SR proteins are imported to the nucleus is conserved between *Drosophila* and humans.

INTRODUCTION

Serine-arginine-rich (SR) proteins are required for constitutive pre-mRNA splicing and also regulate alternative splice

site selection in a concentration-dependent manner. SR proteins have a modular structure that consists of one or two RNA recognition motifs (RRMs) and a C-terminal arginine-serine repeat of varying length (RS domain) (for reviews, see Manley and Tacke, 1996; Graveley, 2000). Functionally, many of the SR proteins are able to bind several classes of specific RNA motifs known as exonic splicing elements, which play a key role in both alternative and constitutive splice site selection in several systems (for reviews, see Tacke and Manley, 1999; Blencowe, 2000). Recently it has been shown that some of the functions of SR proteins can be antagonized by RSF1, a splicing repressor isolated from *Drosophila* that also exhibits a modular organization with a N-terminal RRM-type RNA binding domain and a C-terminal part enriched in glycine (G), arginine (R), and serine (S) residues (GRS domain) (Labourier *et al.*, 1999a,b). The RRM domain of these splicing factors mediates specific recognition of RNA sequences, whereas the RS or GRS domains are responsible for specific protein-protein interactions, which are instrumental for the assembly of the spliceosome (Wu

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Abbreviations used: dTRN, *Drosophila* transportin; dTRN-SR, *Drosophila* transportin-SR; dU1-70K, *Drosophila* U1 small nuclear ribonucleoprotein (snRNP)-70K specific protein; GFP, green fluorescent protein; GRS, glycine-, arginine-, and serine-rich; GST, glutathione S-transferase; HnRNP, heterogeneous nuclear ribonucleoprotein; RRM, RNA recognition motif; RS, arginine- and serine-rich; SR, serine- and arginine-rich; SRPK, SR protein-specific kinase; TRN1, transportin 1; TRN-SR, transportin-SR.

and Maniatis, 1993; Amrein *et al.*, 1994; Kohtz *et al.*, 1994; Zuo and Maniatis, 1996; Labourier *et al.*, 1999b) as well as for the regulation of SR proteins and RSF1 subcellular localization (Li and Bingham, 1991; Hedley *et al.*, 1995; Caceres *et al.*, 1997; this study).

SR proteins can be organized in the interphase nucleus in a characteristic speckled pattern (Fu and Maniatis, 1992; Mintz and Spector, 2000), and also shuttle rapidly and continuously between the nucleus and the cytoplasm (Caceres *et al.*, 1998). This distribution between cellular compartments is expected to alter the steady-state concentrations of SR proteins and thus affect the pattern of alternative splicing. Recently, new members of the importin β (*imp* β) family, termed transportin (TRN)-SR1 and TRN-SR2, have been shown to interact with human SR proteins (Kataoka *et al.*, 1999; Lai *et al.*, 2000, 2001). TRN-SR1 and TRN-SR2 have almost identical sequences except that TRN-SR1 contains additional unique regions in the central and C-terminal parts of the protein. Interaction between TRN-SR1/2 and SR proteins involves the RS domain and is abolished by RanGTP (Lai *et al.*, 2000, 2001). Accordingly, a truncated TRN-SR2 that is defective in Ran binding, colocalizes with SR proteins in nuclear speckles (Lai *et al.*, 2000). It is known that direct interaction of *imp* β with specific nucleoporins mediates docking of the cargo complex to the cytoplasmic face of the nuclear pore complexes, whereas its interaction with RanGTP releases the cargo in the nucleus (for review, see Mattaj and Englmeier, 1998; Izaurralde and Adam, 1998; Pemberton *et al.*, 1998; Gorlich and Kutay, 1999).

The mechanisms regulating the cellular localization of SR proteins are determined, at least in part, by the phosphorylation status of the RS domain. Remarkably, TRN-SR2 has a strong preference for phosphorylated RS domains and mediates nuclear import of phosphorylated, but not unphosphorylated, SR proteins (Lai *et al.*, 2000, 2001). Phosphorylation of serine residues in the RS domain releases these factors from storage/assembly loci (nuclear speckles) and recruits them to the sites of active transcription (Misteli *et al.*, 1998). Furthermore, overexpression of either SR protein kinase (SRPK) or Clk/Sty, a prototypical kinase with dual specificity, capable of phosphorylating tyrosines as well as serines and threonines (Colwill *et al.*, 1996), causes cytoplasmic accumulation of SR proteins (Gui *et al.*, 1994; Colwill *et al.*, 1996). The *Drosophila* homolog of human SF2/ASF (dASF), which lacks phosphorylation sites for *Drosophila* SRPK (dSRPK) in the RS domain, was unable to shuttle between the nucleus and the cytoplasm, although it was imported to the nucleus (Allemand *et al.*, 2001). Because several *Drosophila* SR proteins have distinctive features in their RS domain compared with their human ortholog proteins (Allemand *et al.*, 2001), it is unknown whether the phosphorylation-mediated cellular localization is conserved between the two species. It is also unknown whether the GRS domain of RSF1, like the RS domain, mediates import of RSF1 to the nucleus. In this study, we demonstrate that both the RS domain of *Drosophila* SR proteins and the GRS domain of RSF1 serve as unique nuclear localization signals. We identified a *Drosophila* *imp* β family protein (dTRN-SR) homologous to human TRN-SR2 that specifically interacts with both human and *Drosophila* SR proteins as well as RSF1 and serves as the nuclear import

receptor for many SR proteins and their antagonist RSF1 in *Drosophila*.

MATERIALS AND METHODS

Computer Analysis

BLAST search in the Berkeley *Drosophila* Genome Project (BDGP) database for expressed sequence tags (Rubin *et al.*, 2000) that have homologies with human TRN-SRs revealed a cDNA clone (LD21546) encoding dTRN-SR (GenBank accession number AAF51214). This cDNA is encoded by single-copy gene annotated in the GadFly genomic sequences of the BDGP database as CG2848. The cytological position of this gene is 23B1 on the left arm of chromosome II.

Expression of GFP Fusion Proteins in HeLa and *Drosophila* S2 Cells

Humanized GFP (pEGFP-C1; CLONTECH, GenBank accession number "U55762") was fused in frame to the NH₂ terminus of cDNAs corresponding to dTRN-SR [*Bam*HI-*Eco*RI fragment from pBluescript II (KS+)-dTRN-SR plasmid, described below, inserted between the *Bgl*II and *Eco*RI sites of the vector] and dTRN-SRAN217 (*Pml*I-*Eco*RI fragment from pBluescript II (KS+)-dTRN-SR, inserted between *Hind*III-blunt ended with Klenow enzyme and *Eco*RI sites of the vector).

The pEGFP-hSF2/ASF, pEGFP-hSF2/ASF- Δ RS, pEGFP-dASF, pEGFP-dASF- Δ RS, pEGFP-Rbp1, and pEGFP-B52 constructs were described previously (Allemand *et al.*, 2001). To allow for expression of GFP-B52- Δ RS and GFP-Rbp1- Δ RS, cDNA fragments spanning codons 1–186 and 1–81, respectively, were generated by polymerase chain reaction (PCR) from pUAS-B52 and pGST-Rbp1 plasmids (Labourier *et al.*, 1999b) and cloned in pEGFP-C1 vector between *Bgl*II and *Apa*I sites for B52 and between *Eco*RI and *Bam*HI sites for Rbp1. The pEGFP-hSC35 and pEGFP-hSC35- Δ RS constructs were generated by subcloning the *Eco*RI-*Bam*HI fragments of pUHD-HA-SC35 and pUHD-HA-SC35 Δ RS plasmids (Sureau *et al.*, 2001) into pEGFP-C1 vector. The pEGFP-dSC35 and pEGFP-dSC35- Δ RS constructs were obtained by inserting the cDNA fragments spanning the full-length coding sequence or codons 1–106 of dSC35 (*Drosophila* SC35; generated by PCR amplification of cDNA clone LD32469) between *Eco*RI and *Bam*HI sites of pEGFP-C1 plasmid. The pEGFP-d9G8 and pEGFP-d9G8- Δ RS constructs were obtained by inserting the cDNA fragments spanning the full-length coding sequence or codons 1–81 of d9G8 (*Drosophila* 9G8; generated by PCR amplification of cDNA clone LD02483) in the *Xho*I site of pEGFP-C1 vector. The pEGFP-RSF1 and pEGFP-RSF1 Δ RS were obtained by inserting the cDNA fragments spanning the full-length coding sequence or codons 1–80 of RSF1 (generated by PCR amplification of pGST-RSF1 plasmid (Labourier *et al.*, 1999b) between *Xho*I-*Sal*I and *Xho*I-*Hind*III of the pEGFP-C1 vector, respectively.

The pEGFP-RS(SF2/ASF) and pEGFP-RS(dASF) were described previously (Allemand *et al.*, 2001). To produce green fluorescent protein (GFP) fusions with the RS domains of B52 (aa 187–350), 9G8 (aa 82–259), dSC35 (aa 105–196), and Rbp1 (aa 82–136), and GRS domain of RSF1 (aa 82–201), fragments containing *Hind*III (5' end) and *Bam*HI (3' end) restriction sites (*Xho*I-*Hind*III sites for RSF1) were generated by PCR amplification from appropriate pEGFP constructs described above, and inserted into the pEGFP-C1 plasmid. The RS domain of hSC35 (aa 89–221) was generated by PCR as an *Eco*RI-*Bam*HI fragment and cloned into the appropriate sites of the pEGFP-C2 vector.

All open reading frames and in-frame fusions were entirely sequenced to verify their integrity. Sequences of the oligonucleotides used for all PCR amplifications are available upon request.

Monolayer *Drosophila* S2 or HeLa cells were grown in Schneider's *Drosophila* medium (Invitrogen, Carlsbad, CA) or in RPMI 1640 medium (Invitrogen), respectively, supplemented with 10% fetal

calf serum on 3-cm-diameter dishes (Nalge Nunc, Naperville, IL) to 70–80% confluence. They were transfected with 1 μ g of the indicated plasmids and 19 μ g carrier DNA, by using FUGENE 6 transfection reagent (Roche Applied Science, Indianapolis, IN) for HeLa cells or as indicated by the manufacturer (Kit DES; Invitrogen) for S2 cells. Twenty-four hours posttransfection, cells were washed with phosphate-buffered saline (PBS) and fixed for 15 min at room temperature with 4% paraformaldehyde. The fixed cells were then permeabilized with PBS/0.5% Triton X-100 during 5 min, washed two times with PBS, and then stained either with 4,6-diamidino-2-phenylindole (DAPI) or with a monoclonal antibody (mAb) against SC35 as described previously (Fu and Maniatis, 1992). Each experiment was reproduced in multiple independent transfections, and the cells shown in Figures 1 and 3 are representative of the large effects observed under each set of conditions.

Yeast Strains, Plasmids, and Transformation

To express mutant recombinant proteins deleted in their C-terminal RS or GRS domain by using the yeast pB42AD vector (CLONTECH, Palo Alto, CA), fragments of cDNAs spanning amino acid positions 1–195 for hSF2/ASF (from pEGFP-hSF2/ASF plasmid), 1–189 for dASF (from pEGFP-dASF plasmid), 1–186 for B52 (from pUAS-B52 plasmid; Labourier *et al.*, 1999b), 1–104 for dSC35 (cDNA clone LD32469), 1–81 for d9G8 (cDNA clone LD02483), 1–81 for Rbp1 (from the pEGFP-Rbp1 plasmid) and 1–80 for RSF1 (from the pGST-RSF1 plasmid; Labourier *et al.*, 1999a) were generated by PCR amplification. Except for B52 and RSF1, all DNA fragments contained the *EcoRI* site joined to the ATG start codon at the 5' end and the *XhoI* site joined to the TAA stop codon at the 3' end. Each full-length cDNA or corresponding Δ RS fragment was inserted between *EcoRI* and *XhoI* sites of the pB42AD vector. B52 cDNA and its Δ RS fragment were amplified with oligonucleotides possessing the *SalI* site both at the 5' and 3' ends. RSF1 cDNA and its Δ RS fragment were generated using primers containing the *XhoI* site at both ends. B52 and RSF1 full-length cDNA and their Δ RS fragments were inserted into pB42AD vector digested by *XhoI*.

To express GFP fusions in yeast, *EcoRI-XhoI* fragments obtained from appropriate pB42AD constructs containing full-length cDNAs encoding hSF2/ASF, dASF, dSC35, d9G8, RBP1, and dU1–70K were inserted between the *EcoRI* and *XhoI* sites of pUG36 (URA3 CEN) vector (Bordonne, 2000). RSF1 was inserted as an *XhoI/XhoI* fragment. All GFP-fusions were expressed from an inducible *MET25* promoter. To avoid overexpression, yeast cells were grown under repressing conditions (presence of methionine in the medium) because repression of the *MET25* promoter results in a rest activity of 10–20% (Mumberg *et al.*, 1994).

To obtain a c-myc-tagged dTRN-SR (pRS422-dTRN-SR), a PCR fragment containing *BamHI* and *XhoI* sites at its 5' and 3' ends, respectively, was amplified by PCR and inserted in frame downstream of the c-myc-tag sequence (10 amino acids) into the pRS422 expression vector (ADE2, 2 μ) under the control of the *MET25* promoter.

The yeast strain *mtr10::HIS3* (*mat α , ade2, his3, leu2, trp1, ura3, mtr10::HIS3*) was described previously (Senger *et al.*, 1998). Wild-type (EGY48) yeast strain was a gift from J. Yeakley (University of California at San Diego, San Diego, CA). Yeast strains were transformed following the lithium acetate method (Hill *et al.*, 1991). Yeast cells expressing recombinant proteins from pB42AD plasmids were grown on appropriate liquid media containing galactose.

mtr 10::HIS3 cells expressing recombinant proteins from pUG36 plasmids were allowed to grow on –His, –Ura-selective plates. Resulting strains were complemented with dTRN-SR expressed from pRS422 plasmid and selected on –His, –Ura, –Ade-selective plates. Yeast strains were propagated in yeast extract-peptone-dextrose and SD media by standard methods.

Western and Far Western Blotting

To generate pBluescript II(KS+)-dTRN-SR, a *BamHI-EcoRI* fragment was amplified by PCR from the cDNA clone LD21546, by using Pfu DNA polymerase (Promega, Madison, WI) and inserted between the corresponding sites of pBluescript II (KS+) vector. The pBluescript II (SK–)-dTRN was the generous gift of A. Norvell (HHMI, Princeton University, Princeton, NJ).

Hexahistidine-tagged SF2/ASF and dASF proteins were produced and purified from *Escherichia coli* (eSF2/ASF and edASF) or from baculovirus infected Sf9 cells (bSF2/ASF and bdASF) as described previously (Allemand *et al.*, 2001).

Yeast whole-cell extracts for Western blot analysis were prepared essentially as described previously (Camasses *et al.*, 1998). HeLa nuclear extracts or *Drosophila* Kc nuclear extracts were prepared under standard conditions as described previously (Labourier *et al.*, 1999a; Allemand *et al.*, 2001). Purified SR proteins, whole-cell extracts or nuclear extracts were separated by SDS-PAGE gel, transferred to nitrocellulose by electroblotting for 2 h in 10 mM 3-(cyclohexylamino)-1-propanesulfonic acid, pH 11, transfer buffer containing 10% methanol. For Western analysis, filters were probed either with anti-hemagglutinin (HA) antibodies (Rat mAb, 3F10; Roche Applied Science) followed by incubation with secondary antibody Rat IgG conjugated to peroxidase (Sigma-Aldrich, St. Louis, MO) or anti-c-myc antibodies 9E10 (kindly provided by D. Mathieu (Institut de Génétique Moléculaire, Montpellier, France) followed by incubation with anti-mouse IgG conjugated to peroxidase (Amersham Biosciences, Piscataway, NJ).

For Far Western analysis, the filters were treated as described previously (Kohtz *et al.*, 1994) and probed with ³⁵S-labeled dTRN-SR or dTRN, produced by TNT reticulocyte transcription translation system (Promega) in the presence of [³⁵S]methionine (Amersham Biosciences). Before incubation with the filters, ³⁵S-labeled probes were treated with RNase A (1 mg/ml) for 30 min.

RESULTS

RS Domain of *Drosophila* SR Proteins and GRS Domain of RSF1 Are Sufficient to Mediate Nuclear Import

To examine the cellular distribution of *Drosophila* SR proteins and their antagonist RSF1, GFP was fused in frame to the amino terminus of each of B52/SRp55, dASF, d9G8, dSC35, Rbp1/SRp20, and RSF1 and the fusion proteins were transiently expressed in *Drosophila* S2 cells (Figure 1). All *Drosophila* SR fusion proteins (Figure 1, 3–7), as well as RSF1 (Figure 1, 8) and human SR proteins SF2/ASF (hASF/SF2; Figure 1, 1) and SC35 (hSC35; Figure 1, 2), were localized in the nucleus of S2 cells. In contrast to mammalian cells, where SR proteins colocalized with both speckles and diffuse pools of splicing factors excluding the nucleoli (Allemand *et al.*, 2001), only a diffuse pattern was seen in S2 cells (Figure 1, 1–8). Fusion proteins lacking the RS or GRS domain (Figure 1, 17–24), like GFP alone, had a broader cellular distribution both in the nucleus and cytoplasm. In contrast, the GFP fusions that harbor the RS or the GRS domain alone (Figure 1, 33–40) displayed the same nuclear distribution as full-length fusion proteins, demonstrating that the GRS domain and the RS domain of all SR proteins from *Drosophila* act as nuclear localization signals. None of these fusions, however, were directed to nuclear speckles. Thus, the GRS domain of RSF1 and the RS domain of *Drosophila* SR proteins are necessary and sufficient for nuclear localization.

Figure 1. Cellular localization of GFP fusion proteins in *Drosophila* S2 Schneider cells. Direct fluorescence of GFP-hSF2ASF (1), GFP-hSC35 (2), GFP-B52 (3), GFP-dASF (4), GFP-d9G8 (5), GFP-dSC35 (6), GFP-Rbp1 (7), and GFP-RSF1 (8); GFP-hSF2ASFΔRS (17), GFP-hSC35ΔRS (18), GFP-B52ΔRS (19), GFP-dASFΔRS (20), GFP-d9G8ΔRS (21), GFP-dSC35ΔRS (22), GFP-Rbp1ΔRS (23), and GFP-RSF1ΔGRS (24) deleted of the RS domain or GRS domain; GFP-hSF2ASFΔRRM (33), GFP-hSC35ΔRRM (34), GFP-B52ΔRRM (35), GFP-dASFΔRRM (36), GFP-d9G8ΔRRM (37), GFP-dSC35ΔRRM (38), GFP-Rbp1ΔRRM (39), and GFP-RSF1ΔRRM (40) deleted of the RRM domain. Fusion proteins were analyzed 20 h after transfection. Expression of fusion proteins was confirmed by immunoblot analysis by using an anti-GFP antibody (our unpublished data). The position of nuclei was confirmed by DAPI staining of the same cells transfected with GFP fusion proteins shown in 1–8 (9–16, respectively), 17–24 (25–32, respectively), and 33–40 (41–48, respectively). Bar, 10 μ m.

Identification and Characterization of *Drosophila* Transportin SR

The RS domains of human SR proteins interact directly with two different nuclear import receptors, TRN-SR1 (Kataoka *et al.*, 1999) and TRN-SR2 (Lai *et al.*, 2000). A BLAST homology search with full-length TRN-SR1 and 2 in the fly database (BDGP database) revealed a 914-amino acid protein that bears significant homology to these nuclear import receptors (39/59% identity vs. similarity) and was therefore named dTRN-SR. Because TRN-SR1 has additional amino acids both in the middle and the C terminus (Figure 2), dTRN-SR was more similar to hTRN-SR2 than hTRN-SR1. Both TRN-SR2 and dTRN-SR have significant similarity with the *Saccharomyces cerevisiae* protein Mtr10p (Kadowaki *et al.*, 1994; 25/44% identity vs. similarity for dTRN-SR), a nuclear import receptor for the mRNA-binding protein Npl3p (Pemberton *et al.*, 1997; Senger *et al.*, 1998).

Comparisons of the cDNA and genomic sequences revealed that dTRN-SR is a single copy gene composed of six exons spaced by five introns. Thus, unlike mammals, *D. melanogaster* seems to have only one nuclear import receptor, dTRN-SR, which is probably responsible for the nuclear localization of all SR proteins.

A Truncated dTRN-SR Mutant Exhibits a Speckled Pattern in Nuclei of HeLa Cells

A transiently expressed TRN-SR2 mutant lacking the N-terminal 281-amino acids, Δ N281, localized predominantly in the nucleoplasm and accumulated in speckled domains in the nuclei of HeLa cells (Lai *et al.*, 2000). To test whether deletion of the N-terminal domain of dTRN-SR could similarly affect its cellular distribution, we fused GFP in frame to the N terminus of full-length dTRN-SR or its truncated version lacking the first N-terminal 217-amino acids, Δ N217, and transiently expressed fusion proteins in either HeLa cells (Figure 3, a–h) or *Drosophila* S2 cells (Figure 3, i–p). In both cell lines, full-length dTRN-SR fusion protein was detected both in the nucleus and the cytoplasm (Figure 3, a and i). In contrast, Δ N217 was localized predominantly in the nucleus (Figure 3, b and j). Immunofluorescence with anti-SC35 antibodies clearly established that a dTRN-SR mutant lacking the N-terminal region localized to the nucleus of HeLa cells in a speckled pattern that coincides with the distribution of the SR protein SC35 (Figure 3, b, d, and f). Altogether, the results show that dTRN-SR has the same localization properties as hTRN-SR2, suggesting that dTRN-SR is the import receptor for *Drosophila* SR proteins.

dTRN-SR Recognizes Both Human and *Drosophila* SR Proteins

To determine the specificity of binding of dTRN-SR to target proteins, we used Far Western blotting (Kohtz *et al.*, 1994). This method was successfully applied to reveal specific interactions between members of the SR protein family and other splicing factors (Wu and Maniatis, 1993; Kohtz *et al.*, 1994) as well as for the study of interactions of yeast ribosomal proteins with their specific karyopherins (Rout *et al.*, 1997). Proteins from HeLa and *Drosophila* Kc nuclear extracts were separated by SDS-PAGE gels, transferred to filters, renatured, and probed with 35 S-labeled dTRN-SR produced

in rabbit reticulocyte lysate (Figure 4, lanes 1 and 5). In HeLa nuclear extracts, dTRN-SR labeled predominantly protein species with apparent molecular weights of ~20, 30, 40, 55, 75, 85, and 130 kDa (Figure 4, lane 1). Except for the 85- and 130-kDa proteins, which were only seen with dTRN-SR, protein bands with identical mobilities also appeared when filters were probed with mAb 104 (Figure 4, lane 3), a mAb specific for the phosphoepitope present in a subset of SR proteins. These data suggested that the 20-, 30-, 40-, 55-, and 75-kDa bands are probably SRp20, SRp30, SRp40, SRp55, and SRp75, respectively. Based on the migration, the 85-kDa band is probably the recently identified serine-arginine-rich splicing regulatory protein, SRrp86, which contains a single N-terminal RRM and two C-terminal RS domains, but is not recognized by mAb 104 (Barnard and Patton, 2000). However, further studies are required to confirm this possibility. Binding of dTRN-SR to human SR proteins correlated with their abundance in the extract, suggesting that dTRN-SR had no preference for binding any of the SR proteins. There was, however, a weak signal at the level of SRp55 and SRp75, probably due to a less efficient renaturation than for the other SR proteins.

Given that TRN-SR2 can directly interact with phosphorylated but not unphosphorylated SR proteins (Lai *et al.*, 2001), we tested whether interactions between dTRN-SR and proteins from HeLa extracts are sensitive to phosphorylation. HeLa nuclear extracts were subjected to phosphatase treatment before Far Western blotting. As expected, mAb 104 failed to detect any protein from treated extracts, implying that dephosphorylation of SR proteins was complete (Figure 4, lane 4). In agreement with the fact that dephosphorylation of SR proteins alters their electrophoretic mobility, SR protein bands detected with 35 S-labeled dTRN-SR from treated extracts, had faster mobilities than those from untreated extracts (Figure 4, compare lanes 1 and 2). Dephosphorylated SR proteins, as well as the 85- and 130-kDa bands showed weaker binding signals, indicating that dTRN-SR preferentially bound phosphorylated versions of these proteins (Figure 4, lane 2).

Immunoblotting of mAb 104 to nuclear extracts from *Drosophila* (Kc) cells revealed that SRp55/B52 is the prominent immunoreactive polypeptide (Figure 4, lane 8), whereas 35 S-labeled dTRN-SR revealed several protein bands with molecular weights of ~26, 34, 55, 79, and 100 kDa. Detection of proteins corresponding to 26–79 kDa was strongly diminished after dephosphorylation (Figure 4, lane 6), demonstrating that dTRN-SR recognized weakly unphosphorylated proteins. Consistent with this result, purified recombinant hSF2/ASF and dASF expressed in a baculovirus system (bSF2/ASF and bdASF), where the phosphorylation of recombinant proteins is expected to take place, bind more efficiently dTRN-SR (Figure 4, lanes 13 and 14) than unphosphorylated versions expressed in bacteria (eSF2/ASF and edASF; Figure 4, lanes 11 and 12). However, dephosphorylation did not alter the intensity of the 100-kDa band, whose identity is presently unknown (Figure 4, compare lanes 5 and 6). The 26–79-kDa protein bands have sizes consistent with those of recently identified SR proteins from *Drosophila*, and therefore are likely to be *Drosophila* SR proteins (see below). The interaction between dTRN-SR and *Drosophila* SR proteins is specific, because probing yeast *S. cerevisiae* whole-cell extracts with 35 S-labeled dTRN-SR did

hTRNSR1 1 CGGAGTCTTQVYQAVQALIHDDPDSGKKEASTFVIGELQRSVIAEISDQVQIQRDIEVSC
hTRNSR2 1 CGGAGTCTTQVYQAVQALIHDDPDSGKKEASTFVIGELQRSVIAEISDQVQIQRDIEVSC
dTRNSR 1 ---MDYTSYDVIYQALISALFQDMPKKEQKANKLLQDFQESYTSIADDELQKQKRLAAAT
ntr10 1 ---MDLQVSDIEFALQCTSSSAQDDENKALQFLEQFORSEYVASEICWETLSKEDFTNAL

hTRNSR1 61 YFAAQTMKKHKIQSYFELPTDASHALDLSLLTHIQMLKDLSPVIITQLALATADLALQH
hTRNSR2 61 YFAAQTMKKHKIQSYFELPTDASHALDLSLLTHIQMLKDLSPVIITQLALATADLALQH
dTRNSR 59 YFAAQTMKKHKIQSYFELPTDASHALDLSLLTHIQMLKDLSPVIITQLALATADLALQH
ntr10 59 LELNIFAAQITLRKKVITYDLSQLEHMLDPQFKDSLITLQLSHNQKLITLQLAYALARLARQF

hTRNSR1 120 PSWKGCTQTLVREYSNDYVTSPLFLEILITLPLPEVHRSKSLRIGAKRRETHEIEDAFISST
hTRNSR2 120 PSWKGCTQTLVREYSNDYVTSPLFLEILITLPLPEVHRSKSLRIGAKRRETHEIEDAFISST
dTRNSR 119 ASKREFINDLLVTLAPHCATVPLLEVLITLPLPEHRSYLRIGAKRRETHEIEDAFISST
ntr10 119 LEIQPIFEIISILLNSSPSTLNFILHILPEETLHIASTSLTEVEFVSRIMELDPIAED

hTRNSR1 180 VYSLHHTCEKAGTD-----EKMKHKVRCGLSFLHGLVLDSEFHAKKLLALFFEVLO
hTRNSR2 180 VYSLHHTCEKAGTD-----EKMKHKVRCGLSFLHGLVLDSEFHAKKLLALFFEVLO
dTRNSR 179 VLKPIHCHCLQREBLDQ-----QRYVHAALITYSALLVHAFFVSHYVLAJTLQAFRAIS
ntr10 178 VLKPIVSCBLLQNTDQWSSSSISIEQLKCHSLSTEFFVPEQLLVQPLINLVFETESM

hTRNSR1 234 QDTSSSELREAAASDCVCSALYAIET-----VETELPLAHQLFQGVLTLETAYHHAVARE
hTRNSR2 234 QDTSSSELREAAASDCVCSALYAIET-----VETELPLAHQLFQGVLTLETAYHHAVARE
dTRNSR 234 LPEPSGKLLDHAECVCAILLCHSHYRQDGAASPESSFEAQLPQKALCHLTPYHLSVAARE
ntr10 238 GNESEDHAEFDSALDGLGVITLRSNTHREQLISALFHLQLLQKALPPLTFDMLPLBEY

hTRNSR1 288 DLDEVLNTRICRIFELCEYFLEKIVCTPGQSLGDLRTLELLLCAGHPQYVEVEYSFNEVY
hTRNSR2 288 DLDEVLNTRICRIFELCEYFLEKIVCTPGQSLGDLRTLELLLCAGHPQYVEVEYSFNEVY
dTRNSR 293 DTDCTNTRICRIFELCEYFLEKIVCTPGQSLGDLRTLELLLCAGHPQYVEVEYSFNEVY
ntr10 298 DDDDLLEGHTRLEFVHAGBAWSVYHSMKED---FFKPHVILVHLITCKKEDLVYSTTFPEVF

hTRNSR1 348 ALGEHLIKTDEVIHGFKAQIQRLLHAAARHCQLEPDHEGVPEETDDGFERRRYSOLV
hTRNSR2 348 ALGEHLIKTDEVIHGFKAQIQRLLHAAARHCQLEPDHEGVPEETDDGFERRRYSOLV
dTRNSR 353 LKSLDGLPQIMEDLITVYFPEHIERLISALFHSQSGDSHGLTIEHNEHFFDREKYSOLV
ntr10 356 HFKQSLVLPRIQYRSKXASDIFYKLLNGIITHQLQTPSGQFSSKEBCKKPKFYNHGGDLV

hTRNSR1 408 KDLIFLHGSHECFAQLISTLE-----EGPPEVYEAFLVFIHAAIAKSVDPKKPFSMAACHN
hTRNSR2 408 KDLIFLHGSHECFAQLISTLE-----EGPPEVYEAFLVFIHAAIAKSVDPKKPFSMAACHN
dTRNSR 413 KDVHATVGSAGAKHGHILG-----APETVSEKAPLIGHVAKHILG-----
ntr10 416 KDCATVGSSEALSQPLIRHISAIHGEVSLQIEAPGESLTHAKISLT-----

hTRNSR1 465 SLLFGQNTITSEISNCEYLPFVIREHEPTVVELEGVNLPEVHTAVRITSILVGEHSE
hTRNSR2 454 -----HEPTVVELEGVNLPEVHTAVRITSILVGEHSE
dTRNSR 459 DEHEVYVVEALHNSBOTHAVRITAILHGLCD
ntr10 466 EN-TIIPREKKIKCHLPQAK--RTASTLVIGRYTE

hTRNSR1 525 VYDNPQFIDPVLGVLKKGCEPK-----LASAAAKAHHCISYCRDHMAQHIFGLE
hTRNSR2 491 VYDNPQFIDPVLGVLKKGCEPK-----LASAAAKAHHCISYCRDHMAQHIFGLE
dTRNSR 496 WIENHPESEALINFLYALQKKG-----LAFAAATALYSICSAQCKHVCHISGLVE
ntr10 500 WTAKNPELVQVQYVYVNSGLNECSSDQSSITASSNALHFFCSQSKLLVYIDQLIN

hTRNSR1 578 IARSLSFLSPPEAAVGLLKGTALVIALRPLDKITCELSBSCSVQVHALKLLSQE-----P
hTRNSR2 544 IARSLSFLSPPEAAVGLLKGTALVIALRPLDKITCELSBSCSVQVHALKLLSQE-----P
dTRNSR 550 IARSLSFLSPPEAAVGLLKGTALVIALRPLDKITCELSBSCSVQVHALKLLSQE-----P
ntr10 560 FFLEYQSS-IDIESQFELCOGLSALHTEQEAIVSVTQKVDDBLRQALIPQW-KAY

hTRNSR1 635 SNGISSDPTVYFLDRLAVIFRHTNP--IVHNGQTHPCQKVIQEIYVPLSEYLNKH--RADN
hTRNSR2 601 SNGISSDPTVYFLDRLAVIFRHTNP--IVHNGQTHPCQKVIQEIYVPLSEYLNKH--RADN
dTRNSR 610 QKGETIDPVYVDRACALHRTNP--DVPDVEHPTVAILNDVQLHSRYHDKY--QSDL
ntr10 618 PTLAPQIADKILHLIALFEELRPNRYNYPQQSGEPLPPIHFIYKARITLVYDAGANTDS

hTRNSR1 691 RIVERCCCLRFAYRCVGGSAALLOPLVTOMVYVYHRSFCFLYLSILVDEHGEHE
hTRNSR2 657 RIVERCCCLRFAYRCVGGSAALLOPLVTOMVYVYHRSFCFLYLSILVDEHGEHE
dTRNSR 666 RIVERTCCLRIYGFIERVQKQAGHLEPLKQOMVYVYHRSFCFLYLSILVDEHGEHE
ntr10 678 RIVERTVAKLLRIFIRFHVFCAPLTPSVAEFLHQMGLTTFGSLVCSGSLVYFGDDHS

hTRNSR1 750 -----SCROGLLDHLQALCIPFFOLLEQQGLQHHPDVTYDDLFELATRFQIQRPTTL
hTRNSR2 716 -----SCROGLLDHLQALCIPFFOLLEQQGLQHHPDVTYDDLFELATRFQIQRPTTL
dTRNSR 725 -----ECIGGLLDHLQAFIEPTFGLQMGSLKLPD-----T-----L
ntr10 738 FPIPSFLKDAVWFALSQCTFHLHKKFBDKQLNDYHEAIDVYFSLISULINHPGAFLL

hTRNSR1 802 LRSQVFIPIQVLAAS--TLDRHDAGSVHFFEDLHGVAND-----HEEDFERK
hTRNSR2 768 LRSQVFIPIQVLAAS--TLDRHDAGSVHFFEDLHGVAND-----HEEDFERK
dTRNSR 759 LRSQVFIPIQVLAAS--TLDRHDAGSVHFFEDLHGVAND-----HEEDFERK
ntr10 798 NSTELLEVLVLAECVHKLDDVDAVICLRCDHDSGFKTFPISTVSIETVPDEHEK

hTRNSR1 854 ELIGQVHHLGQQLVYSLHLTCFCFLPPTLPDAEVLVEIHQYDR-PTFCRVLENSLKG
hTRNSR2 820 ELIGQVHHLGQQLVYSLHLTCFCFLPPTLPDAEVLVEIHQYDR-PTFCRVLENSLKG
dTRNSR 810 PLVYELASQVHGLVGLQGLVYSLHLTCFCFLPPTLPDAEVLVEIHQYDR-PTFCRVLENSLKG
ntr10 858 QVHVEYVANGQLILVYFGLVYTFENFASDAISVYLCRLITTEANNNDACIDWV

hTRNSR1 913 LPKETTIVGAYTVTHK-OLDFHEQVTSAECKQVCTALRDETQVYSIANTPCTCALSPRN
hTRNSR2 870 LPKETTIVGAYTVTHK-OLDFHEQVTSAECKQVCTALRDETQVYSIANTPCTCALSPRN
dTRNSR 879 LPKENSQGVYLAHQ-OLDBESSTLADRTNATSALEKTEFLER
ntr10 918 YKYVEQLSQVLTWERDLAKAVYGLNSKDYKVRGEGIRAVGVWSRKXINSRFE

hTRNSR1 972 VFFM

Figure 2. Amino acid sequence alignments of dTRN-SR with TRN-SR1, TRN-SR2, and Mtr10p as obtained by the ClustalW program. Identical amino acid residues are marked in dark and conservative substitutions such as RKH, IVLM, ED, FY, and ST are marked in gray. Gaps are introduced to optimize amino acid sequence alignments.

Figure 3. Cellular localization of dTRN-SR GFP fusion proteins in HeLa cells (a–h) and in *Drosophila* S2 Schneider cells (i–p). Direct fluorescence of GFP-dTRN-SR (a and i) and GFP-dTRN-SR Δ N217 (b and j) fusion proteins were analyzed 20 h posttransfection. Expression of fusion proteins was confirmed by immunoblot analysis by using an anti-GFP antibody (our unpublished data). The position of nuclei was confirmed by DAPI staining of the same transfected HeLa (g and h) and S2 (k and l) cells with GFP-dTRN-SR (g and k) or GFP-dTRN-SR Δ N217 (h and l). Indirect immunofluorescence staining of HeLa cells in a and b with the α SC35 mAb (c and d, respectively) showed the cellular localization of endogenous SR protein SC35. A merge between a and c (e) and between b and d (f) shows that dTRN-SR Δ N217 and SC-35 colocalize in the speckles. The Normarski interference-contrast of the same S2 cells transfected with GFP-dTRN-SR (o) and GFP-dTRN-SR Δ N217 (p) are shown. Bar, 10 μ m

not reveal any reactive protein (Figure 4, lane 7). Indeed, yeast *S.cerevisiae* does not have SR proteins and mAb 104 does not cross-react with any protein in yeast (Figure 4, lane 10).

To test whether another member of imp β family was capable of interacting with SR proteins, we performed a Far Western experiment with dTRN, an ortholog to human TRN1 and *S. cerevisiae* Kap104p. TRN1 binds the M9 sequence of hnRNP A1 and is responsible for targeting this protein to the nucleus (Pollard *et al.*, 1996), whereas Kap104p plays a role in the nuclear import of a yeast hnRNP-like protein Nab2p (Aitchison *et al.*, 1996). Our data (lanes 15–18) clearly show that protein bands revealed by 35 S-labeled dTRN were different from those detected by dTRN-SR in HeLa and Kc extracts. In *Drosophila* extracts, for example,

there were two sets of proteins that interacted with dTRN, one at $\sim$ 75 kDa, and others between 30 and 40 kDa (Figure 4, lane 17). The latter proteins are probably *Drosophila* hnRNPs, Squid (SqdB and SqdS)/hp40, known to bind dTRN (Norvell *et al.*, 1999). Unlike the interaction between dTRN-SR and SR proteins, the interaction between dTRN and its target proteins was not sensitive to dephosphorylation (Figure 4, lanes 16 and 18). Thus, only dTRN-SR, but not dTRN, binds *Drosophila* SR proteins.

dTRN-SR Binds Specifically to RS Domain of SR Proteins

The Far Western experiments described above demonstrated that dTRN-SR does not bind proteins from yeast whole-cell

Figure 4. Far Western analysis showing a physical interaction between dTRN-SR and SR proteins from both HeLa and *Drosophila* Kc cells. Proteins from HeLa (lanes 1–4, 15, and 16), *Drosophila* Kc (lanes 5, 6, 8, 9, 17, and 18) nuclear extracts or yeast *S. cerevisiae* total extracts (lanes 7 and 10) either untreated (lanes 1, 3, 5, 7, 8, 10, 15, and 17) or treated (lanes 2, 4, 6, 9, 16, and 18) with calf intestinal alkaline phosphatase (CIAP) as well as purified recombinant dASF and SF2/ASF 100 ng each, expressed either in a baculovirus system (bdASF, lane 13, and bSF2/ASF, lane 14, respectively) or in *E. coli* (edASF, lane 12, and eSF2/ASF, lane 11, respectively) were separated on a SDS-PAGE, transferred to nitrocellulose, renatured, and probed either with ^{35}S -labeled dTRN-SR (lanes 1, 2, 5–7, and 11–14) or ^{35}S -labeled dTRN (lanes 15–18). Proteins (lanes 3, 4, 8, and 9) were subjected to Western blot analysis by using mAb 104. Molecular weight markers are indicated on the left of the Far Western panels, and SR protein species are indicated on the right.

extracts. This finding prompted us to use yeast *S. cerevisiae* as model system to examine the potential requirement of different SR proteins domains and eventually RSF1 for the interaction with dTRN-SR. To this end, both wild-type and deletion mutants deleted in the RS domain (ΔRS) of hSF2/ASF and *Drosophila* SR proteins and GRS domain (ΔGRS) of RSF1 were expressed from a multicopy plasmid under control of the GAL10 promoter in yeast. The recombinant proteins contained an N-terminal HA-tag to facilitate their detection with an anti-HA antibody. Western blotting (Figure 5B) of total yeast extracts with an anti-HA antibody confirmed the expression of full-length and deletion mutants in a wild-type yeast strain. Probing duplicated filters with ^{35}S -labeled dTRN-SR revealed that dTRN-SR bound efficiently to hSF2/ASF, dASF, dSC35, d9G8, Rbp1, and B52, but only weakly to RSF1 (Figure 5A, compare lanes 1, 3, 5, 7, 9, 11, and lane 13). In sharp contrast, none of the mutant proteins deleted in the RS domain (hSF2/ASF ΔRS , dASF ΔRS , dSC35 ΔRS , d9G8 ΔRS , Rbp1 ΔRS , and B52 ΔRS) or GRS domain (RSF1 ΔGRS) were detected with ^{35}S -labeled dTRN-SR, showing that the RS domain is required for interaction between dTRN-SR and SR proteins. The lack of recognition between dTRN-SR and proteins deleted in the RS domain or GRS domain provides further evidence that the observed interaction between dTRN-SR and the SR proteins or RSF1 is specific.

dTRN-SR Serves as an Import Receptor for Drosophila SR Proteins and RSF1 In Vivo

The RNA binding protein Npl3p that has been implicated in mRNA transport (Lee *et al.*, 1996) is one of the yeast proteins, that resembles both hnRNP and SR proteins from mamma-

Figure 5. Binding of dTRN-SR to SR proteins, RSF1 and deletion mutants expressed in wild-type yeast strains. Total extracts prepared from yeast expressing indicated recombinant proteins (lanes 1–14) were assayed by either Far Western analysis by using ^{35}S -labeled dTRN-SR (A) or Western blot analysis by using 3F10 anti-HA antibody (B). Both panels represent two independent migrations.

Figure 6. Nuclear import of SR proteins and RSF1 requires dTRN-SR in yeast cells. Direct fluorescence of GFP-hSF2/ASF, GFP-dASF, GFP-dSC35, GFP-Rbp1, GFP-d9G8, GFP-RSF1, GFP, GFP-dU1-70K, and GFP-NPL3 was analyzed after transformation in either *mtr10::HIS3* cells (A) or *mtr10::HIS3* cells complemented with c-myc-dTRN-SR (B). The position of nuclei was confirmed by DAPI staining of the transformed cells. Bar, 5 μ m.

lian cells (Siebel *et al.*, 1999; Gilbert *et al.*, 2001). Curiously, the nuclear import receptor for Npl3p was identified as Mtr10p, the yeast relative of dTRN-SR (Senger *et al.*, 1998). Thus, it is possible that SR proteins in yeast use Mtr10p as a nuclear import receptor (Yeakley *et al.*, 1999). Given that deletion of *MTR10* resulted in cytoplasmic accumulation of Npl3p (Pemberton *et al.*, 1997; Senger *et al.*, 1998), the latter scenario predicts that in the absence of Mtr10p, SR proteins and potentially RSF1 should also accumulate in the cytoplasm. We tested this hypothesis by expressing GFP-SR fusion proteins in an *MTR10* deletion mutant. To avoid overexpression of these fusion proteins, they were expressed using a centromeric plasmid without induction (see MATERIALS AND METHODS). The levels of expression were monitored by Western blot analysis with an anti-GFP antibody (our unpublished data). When living cells were analyzed by fluorescence microscopy, GFP-SF2/ASF, GFP-dASF, GFP-dSC35, GFP-Rbp1, GFP-d9G8, GFP-RSF1, and GFP accumulated in the cytoplasm of the *mtr10* mutant (Figure 6A). Similar cytoplasmic localizations were observed in wild-type cells (our unpublished data), indicating that the lack of Mtr10p was not responsible for the cytoplasmic accumulation of SR proteins. As expected, GFP-Npl3 also had a cytoplasmic distribution in the *mtr10* mutant (Figure 6A), whereas it had a nuclear localization in wild-type cells (our unpublished data). As a control, we analyzed the local-

ization of *Drosophila* U1-70K fused to GFP (Figure 6A). dU1-70K, a specific protein associated with U1 snRNP, contains a classical nuclear localization signal (Romac *et al.*, 1994), and as such its localization should not be affected by the *MTR10* deletion. As expected, no inhibition of nuclear import of this protein was observed in either the *mtr10* mutant (Figure 6A) or wild-type cells (our unpublished data), again showing that Mtr10p could not replace dTRN-SR for nuclear import of *Drosophila* SR proteins exogenously expressed in yeast. These results are also consistent with the idea that other nuclear import receptors, like Kap104, the functional homolog of dTRN (Aitchison *et al.*, 1996; Siomi *et al.*, 1998), cannot replace dTRN-SR to mediate nuclear localization of SR proteins in yeast. Moreover, none of the SR proteins expressed in yeast were able to bind dTRN, as revealed by Far Western (our unpublished data).

To test whether mislocalization of GFP-SR protein fusions in yeast could be corrected by dTRN-SR, *mtr10* mutants expressing *Drosophila* SR proteins, hSF2/ASF as well as RSF1 were transformed with plasmids expressing c-myc-tagged dTRN-SR. Immunoblotting with anti-c-myc antibodies revealed that similar levels of the c-myc-dTRN-SR fusion were expressed (our unpublished data). As shown in Figure 6B, expression of c-myc-dTRN-SR in *mtr10* mutant strains induced dramatic changes in the localization of GFP-*Drosophila* SR proteins (Figure 6B), hSF2/ASF (Figure 6B, 1) as

well as RSF1 (Figure 6B), which exhibited an exclusive intranuclear location in the complemented strains. However, the distribution of GFP (Figure 6B), dU1-70K (Figure 6B) and Npl3 (Figure 6B) remained unchanged. Taken together, these data show that dTRN-SR specifically mediates the nuclear import of *Drosophila* SR proteins as well as their antagonist RSF1.

DISCUSSION

In this report we identify the *Drosophila* dTRN-SR, homologous to human TRN-SR2, as a nuclear import receptor for both *Drosophila* SR proteins and their antagonist RSF1. The extensive conservation between vertebrate and *Drosophila* dTRN-SR supports the hypothesis that an important event in the regulation of splicing takes place at the level of nuclear uptake of SR proteins and their antagonist. Considering that the SR- and RSF1-related proteins can affect splice site selection in a concentration-dependent manner, the regulation of this nuclear traffic of splicing factors may play an important role in the regulation of alternative splicing. Previous studies showed that protein kinases that modify the RS domain of SR proteins may contribute to their spatial and temporal regulation (Tazi *et al.*, 1997), as well as to the modulation of their activity (Wang and Manley, 1997). In this context, it is significant that phosphorylation of these factors alters their interaction with dTRN-SR. Using far Western experiments, we have been able to show that both human and *Drosophila* SR proteins interact with dTRN-SR in a phosphorylation-dependent manner. Like the mammalian TRN-SR2, dTRN-SR preferentially associates with phosphorylated SR proteins. Thus, these results demonstrate the high degree of evolutionary conservation of function of the transportin pathway and corroborate previous work in mammalian cells showing that phosphorylation of the RS domain is an important determinant in the nuclear uptake of SR proteins (Yeakley *et al.*, 1999; Lai *et al.*, 2000, 2001).

Although TRN-SR2 was only shown to interact with SR proteins, this study provides the first evidence that the *Drosophila* homolog dTRN-SR is able to bind both SR proteins and the splicing repressor RSF1. The most prominent feature of the GRS domain of RSF1, which mediates interaction with dTRN-SR, is its abundance in Gly, Ser, and Arg, but its primary sequence is not significantly similar to the RS domain of the SR protein. The observed interaction of dTRN-SR with the GRS domain strengthens the idea that dTRN-SR recognizes its import substrates not only via a primary sequence but also by secondary and/or tertiary structural features. Consistent with this idea, the RS domains of individual vertebrate SR proteins are conserved only in terms of their overall composition and the presence of many consecutive RS or SR dipeptides, whereas several *Drosophila* SR proteins have a glycine hinge region between the RNA binding domain [with one or two RRM(s)] and the RS repeats. Significantly, the *Drosophila* homologue of hSF2/ASF, dASF, which lacks the RS repeats and instead has a G-rich region at the RS domain (Allemand *et al.*, 2001), interacted with dTRN-SR and was efficiently imported in yeast complemented with dTRN-SR (Figures 4 and 6). Furthermore, although phosphorylation of the RS domain by SRPK1 has been shown to be critical for efficient nuclear import of SR fusion proteins via TRN-SR2, this does not

seem to apply for dTRN-SR. RSF1 and dASF, for instance, which are not substrates for SRPK kinases from different organisms (Allemand *et al.*, 2001; our unpublished data), interacted with dTRN-SR in a RS- or GRS-domain-dependent manner and were efficiently imported both in S2 and yeast cells, provided that the cells contained dTRN-SR. This result is consistent with recent reports showing that phosphorylation by SRPKs promotes shuttling of SR proteins between the nucleus and cytoplasm (Allemand *et al.*, 2001; Gilbert *et al.*, 2001). Thus, the mechanism by which dTRN-SR recognizes its target proteins is of considerable interest and remains to be clarified. The yeast system may be useful for analyzing the phosphorylation sites of RS or GRS domains at different levels of SR kinases and for examining whether differentially phosphorylated RS or GRS domains exhibit different affinities to dTRN-SR.

Another striking result of our analysis was the finding that the RS domain of all SR proteins tested, was sufficient to trigger nuclear localization in S2 cells, whereas in mammalian cells SR proteins containing two RRMs, such as SF2/ASF, dASF and B52/SRp55, did not require the RS domain for proper nuclear localization (Allemand *et al.*, 2001). It is therefore possible that in mammalian cells, there are at least two nuclear import pathways for SR proteins: RS-dependent and RS-independent pathways. This could explain why mammalian cells contain two transportins, TRN-SR1 and TRN-SR2, mediating nuclear import of SR proteins. Given that TRN-SR2 is capable of targeting phosphorylated but not unphosphorylated SR proteins to the nucleus (Lai *et al.*, 2001) and that TRN-SR1 does not seem to have such a requirement (Kataoka *et al.*, 1999), it is tempting to speculate that TRN-SR1 is responsible for the import of SR proteins regardless of phosphorylation and/or presence or absence of the RS domain. In contrast, *Drosophila* cells only have one transportin for both SR proteins and RSF1, which do not have a classical RS domain.

During initial steps of the spliceosome assembly, the RS or GRS domain of SR proteins and RSF1 are used for a variety of protein-protein interactions, which play crucial roles in splice site selection. Herein, we have shown that these domains can also mediate a specific interaction with dTRN-SR. Our Far Western experiments demonstrate that dTRN-SR has the ability to interact with multiple RS-containing proteins from two highly divergent species, *Drosophila* and human. However, we noted a weak binding of dTRN-SR to human SRp75, when this protein was abundant. A similar result was also obtained with TRN-SR1 (Kataoka *et al.*, 1999), suggesting that SRp75 either has a different receptor or is difficult to renature under the conditions used for Far Western analysis. We favor the latter possibility because Far Western experiments performed with labeled SR proteins also showed weaker binding to SRp75 compared with other SR proteins (Labourier *et al.*, 1999b; Barnard and Patton, 2000).

Thus, this work paves the way toward a molecular genetic analysis of the biological role of dTRN-SR, which may allow the elucidation of factors modifying the activity of SR proteins. The interaction between dTRN-SR and target proteins is so robust that it can be used to screen expression libraries with ³⁵S-labeled dTRN-SR. Among potential candidates, are the 100- and the 85-kDa proteins identified from *Drosophila* Kc (Figure 4, lanes 5 and 6) and HeLa (Figure 4, lanes 1 and

2) nuclear extracts, respectively. Given that SR proteins and their antagonist RSF1 are specific targets for dTRN-SR, we may identify novel factors that regulate splice site selection by regulating the effects of some SR proteins. Further studies designed to determine whether the 85-kDa band corresponds to SRp86, which has such a function in splicing, should confirm this prediction.

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