A Human Importin-β Family Protein, Transportin-SR2, Interacts with the Phosphorylated RS Domain of SR Proteins*

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Serine/arginine-rich proteins (SR proteins) are mainly involved in the splicing of precursor mRNA. RS domains are also found in proteins that have influence on other aspects of gene expression. Proteins that contain an RS domain are often located in the speckled domains of the nucleus. Here we show that the RS domain derived from a human papillomavirus E2 transcriptional activator can target a heterologous protein to the nucleus, as it does in many other SR proteins, but insufficient for localization in speckles. By using E2 as a bait in a yeast two-hybrid screen, we identified a human importin-β family protein that is homologous to yeast Mtr10p and almost identical to human transportin-SR. This transportin-SR2 (TRN-SR2) protein can interact with several cellular SR proteins. More importantly, we demonstrated that TRN-SR2 can directly interact with phosphorylated, but not unphosphorylated, RS domains. Finally, an indirect immunofluorescence study revealed that a transiently expressed TRN-SR2 mutant lacking the N-terminal region becomes localized to the nucleus in a speckled pattern that coincides with the distribution of the SR protein SC35. Thus, our results likely reflect a role of TRN-SR2 in the cellular trafficking of phosphorylated SR proteins.

SR proteins are a superfamily of eukaryotic proteins that contain repetitive serine-arginine dipeptides in a domain known as RS domains (1–3). SR proteins are primarily involved in the splicing of precursor mRNA. Some SR splicing factors are essential for pre-mRNA splicing, and some can act as crucial players in alternative splicing by modulating splice site choice. A group of SR proteins that can be precipitated by magnesium and recognized by monoclonal antibody (mAb) 104 play both essential and regulatory roles in pre-mRNA splicing (4). Each of these SR proteins can complement splicing-deficient cytoplasmic S100 extracts as well as affect splice site selection at elevated concentrations (1–3). In addition to splicing factors, RS domains are present in other proteins, such as a group of human papillomavirus E2 transcriptional activators (5, 6), RNA pol II-associated SR-like proteins (7), transcriptional coactivator PGC-1 (8), and pre-mRNA cleavage factor Im (9). Thus, RS domain-containing proteins can function in gene expression at different levels.

Cytological studies have revealed that a variety of SR proteins are localized in nuclear speckled domains, which are thought to be the sites for storage/reassembly of splicing factors and/or supplying splicing factors to active genes (10, 11). The RS domains of some, but not all, SR proteins have been shown to be necessary and sufficient for targeting to the nuclear speckles (12, 13). Hyperphosphorylation of the RS domain by SR protein-specific kinases can relocate SR proteins from a speckled pattern into a more diffuse distribution (14–16). Recent evidence indicates that a subset of SR proteins can continuously shuttle between the nucleus and the cytoplasm (17). The phosphorylation states of the RS domain appear to have influence on the shuttling properties of SR proteins (17). Thus, the nucleocytoplasmic transport and nuclear speckle localization of SR proteins are likely to be complex and regulated processes.

Translocation of macromolecules across the nuclear envelope occurs through the nuclear pore complex (18–21). Proteins targeted for the nucleus are initially complexed with corresponding soluble import receptors in the cytoplasm via specific signal-receptor recognition. Receptor-cargo complexes subsequently dock at saturable sites on the cytoplasmic face of the nuclear pore and then translocate through the pore to the nuclear interior. Nuclear translocation of cargo requires additional factors such as the small GTPase Ran and NTF2 (18–21). Different import cargoes possess different nuclear localization signals (NLSs) (18, 21). The prototypical NLS is composed of one or more clusters of basic amino acid residues and is recognized by importin-α, which functions as an adapter and in turn interacts with importin-β for nuclear pore complex docking. Nonclassic NLSs include the glycine-rich M9 sequence of heteronuclear ribonucleoprotein A1 (22, 23), the arginine-rich sequence of human immunodeficiency virus regulatory proteins Rev and Tat (24, 25), and the RGG box of the yeast RNA-binding protein Npl3p (26, 27). These NLSs, unlike the prototypical NLS, interact directly with their corresponding import receptors. All of the import receptors belong to the importin-β family. They are of similar size (90–130 kDa) and appear to consist of 18 or more helix-turn-helix HEAT repeats as revealed by the crystal structures of two importin-β proteins (28–30). It is well known that the N- and C-terminal halves of the importin-β proteins contribute to interaction with GDP-bound Ran and the NLS of cargo, respectively. However, importin-β interactions with Ran and NLSs are mutually exclusive, implying the existence of a RanGTP-mediated cargo release mechanism.

We previously showed that a RS domain-containing human papillomavirus (type 5) E2 transcriptional activator can func-
tion to facilitate the splicing of pre-mRNA made via transactivation by E2 itself (6). This E2 transactivator colocalizes with cellular splicing factors in nuclear speckles, and its RS-rich hinge domain is required for colocalization (6). In the present study, we establish that the E2 hinge can target a heterologous protein to the nucleus but not to subnuclear speckle domains. To understand the mechanism of RS domain-mediated nuclear entry and its regulation, we searched for factors that could play a role in the nuclear trafficking of SR proteins. A human importin-β family protein was identified and its interactions with the phosphorylated SR proteins characterized.  

**Experimental Procedures**

**Plasmid Construction**—The β-gal-hinge and β-gal-RS expression vectors were constructed by insertion of a PCR product derived from the entire hinge region (amino acids 212-296) or the RS-rich subdomain (amino acids 212-346) of HPV-5 E2 into the unique PstI site within pCH110 (Amersham Pharmacia Biotech), respectively. The resulting fusion proteins expressed in HeLa cells can be detected by monoclonal anti-β-gal antibody. The SRPK1 expression vector that produced FLAG-tagged SRPK1 in HeLa cells was kindly provided by X.-D. Fu (University of California, San Diego, CA) (16). Plasmid pGAD-TRN-SR2C was obtained by subcloning the DNA fragment encoding the C-terminal 399 amino acids of the human TRN-SR2 protein into *Escherichia coli* expression vector pGEX-2T (Amersham Pharmacia Biotech). The resulting plasmid was used to produce the recombinant GST-TRN-SR2C protein in bacteria. ASF and SRPK1 open reading frames (gifts of X.-D. Fu) were also inserted into pGEX-2T to generate plasmids encoding GST-ASF and GST-SRPK1 fusion proteins, respectively. The pBluescript-derived plasmids encoding full-length and ΔN281 TRN-SR2 were constructed by appropriate restriction digestion of the parental pBluescript plasmid containing a 3.4-kilobase pair TRN-SR2 cDNA (see “Results”) to remove the 5'-untranslated region and 5'-untranslated region plus a region coding for the N-terminal 281 amino acid residues, respectively. The resulting plasmids generated in-frame fusion of TRN-SR2 to the first 34 amino acids of β-gal at the N terminus.  

**Indirect Immunofluorescence**—Cell culture, transfection, and indirect immunofluorescence staining were performed essentially according to Lai et al. (6). For treatment of cells with an RNA pol II inhibitor, transfected cells were incubated with 100 μM DRB for 4 h before fixation. The primary antibodies used included monoclonal anti-β-gal antibody (2 μg/ml; Promega), purified sheep anti-rabbit IgG (7.5 μg/ml; Cappel Laboratories), monoclonal anti-HA antibody (1:100 dilution from the supernatant of hybridoma culture medium; gift of S.-C. Cheng, Academia Sinica, Taipei, Taiwan), polyclonal anti-HA antibodies (1:50 dilution; Upstate Biotechnology Inc., Lake Placid, NY), and monoclonal anti-SC35 antibody (4.6 μg/ml; Sigma). The secondary antibodies used were fluorescein-conjugated anti-mouse IgG (7.5 μg/ml; Cappel Laboratories) for monoclonal primary antibodies and streptavidin-conjugated anti-rabbit IgG (12 μg/ml; Cappel Laboratories) for polyclonal primary antibodies. The specimens were observed using a laser confocal microscope (MRC 600 model; Bio-Rad) coupled with an image analysis system.

**Yeast Two-hybrid System and cDNA Cloning**—HPV-5 E2 was cloned in frame into the GAL4 DNA binding domain (DB) plasmid pAS2-1 and then used as a bait to screen a HeLa cell cDNA library (CLONTECH) that was constructed in the GAL4 activation domain (AD)-containing pGAD GH vector. Yeast two-hybrid screening was performed as described in the protocol provided by the manufacturer. Initially, the bait plasmid was transformed into Saccharomyces cerevisiae Y190 and maintained by selection in Trp medium. The cDNA library was then transformed into the bait-containing yeast cells, and transformants were selected by the use of appropriate media. One of the positive clones, which encoded the C-terminal 399 amino acids of a human importin-β family protein, was named pGAD-TRN-SR2 (see “Results”). To obtain cDNAs encoding full-length TRN-SR2, the insert of pGAD-TRN-SR2 was used as a probe to screen a λZAP cDNA library made from HeLa cells (CLONTECH). The cDNA inserts of positive clones were excised into pBluescript phagemids as described in the manufacturer’s instruction, and the sequences were then determined by automated sequencing. The TRN-SR2 coding sequence matched perfectly to GenBank™ accession number AJ133769. To assay for pairwise interactions between TRN-SR2 and SR proteins, pGAD-TRN-SR2 and a pEG202-derived plasmid expressing individual SR proteins (gifts of J. Y. Wu, Washington University, St. Louis, MO) or domains as LexA fusion proteins were co-transformed into reporter pSH18-34 containing EGY48. The method of the liquid β-galactosidase assay was described in the CLONTECH protocol. To examine whether phosphorylation of the RS domain is important for the TRN-SR2-SR protein interaction, pGAD-TRN-SR2 was co-transformed with pGAD-ASF or pEG-SR2C into EGY48, corresponding SKY1 strain, the known yeast transformants were selected by the use of appropriate media. One of the positive clones was excised into pBluescript phagemids as described in the manufacturer’s instruction (Amersham Pharmacia Biotech). The purified fusion protein was subjected to cleavage by thrombin protease followed by preparative gel electrophoresis. Gel-purified TRN-SR2 C-terminal domain was then used to raise antisera in rabbits. To purify anti-SR-SR2 antibodies, antisera were incubated with nitrocellulose containing immobilized GST-TRN-SR2C protein at room temperature overnight. Antibodies were eluted from the filters with a solution containing 50 mM glycine (pH 2.3) and 150 mM NaCl, followed by neutralization with Tris base.

**Expression and Purification of Recombinant Proteins**—His-tagged wild-type Ran and RanQ69L (gifts from I. W. Mattaj, European Molecular Biology Laboratory, Heidelberg, Germany) were expressed in *E. coli* strain BL21/pRep4 and purified on nickel-nitrilotriacetic acid (Novagen) essentially according to Gorlich et al. (33). Purified Ran and RanQ69L were initially dialyzed against 50 mM potassium phosphate (pH 7.0), 50 mM KCl, 5 mM MgCl2, 1 mM β-mercaptoethanol, 8.7% glycerol, and 0.1 mM GDP (for Ran) or 0.1 mM GTP (for RanQ69L). Before use, they were loaded with 1 mM GDP and GTP, respectively, according to Floer and Blobel (34). GST-ASF and GST-SRPK1 were overexpressed in *E. coli* strains BLR and XA90, respectively, upon isopropyl-1-thio-β-D-galactopyranoside induction. The two GST fusion proteins were purified using glutathione-Sepharose, and then dialyzed against buffer D containing 20 mM HEPEs (pH 7.9), 50 mM KCl, 0.2 mM EDTA, 1 mM dithiothreitol, and 20% glycerol. Recombinant full-length and ΔN281 TRN-SR2 proteins were expressed in *E. coli* strain XA90 after induction with isopropyl-1-thio-β-D-galactopyranoside. The extracts were prepared by lysis of cells with modified transport buffer (35) containing 20 mM HEPEs (pH 7.3), 110 mM potassium acetate, 2 mM magnesium acetate, 5 mM sodium acetate, 2 mM dithiothreitol, 1 mM EGTA, 8.7% glycerol, and 1 mM phenylmethylsulfonfyl fluoride. The lysates were stored in aliquots after removal of cell debris.

**Expression of SRPK1 in *E. coli* Cell Cytoplasmic Extract**—HeLa cells (strain S3) were cultured in RPMI supplemented with 10% fetal bovine serum and 1% penicillin-streptomycin at a density of 5 × 10^6 cells/ml. The cytoplasmic extract was prepared from HeLa cells essentially according to Paschal (36), and finally dialyzed against the transport buffer supplemented with 1 μM each of aprotinin and leupeptin. The concentration of the cytoplasmic extract was ~25 mg/ml.

**In Vitro Pull-down Assay**—In vitro phosphorylation of GST-ASF was carried out in a 20-μl mixture containing 2 μg of GST-ASF, 2 mM MgCl2, 0.5 mM ATP (or 0.5 mM ATP-γ-S), and 30 ng of GST-SRPK1 at 30 °C for 45 min. ATP was eliminated in the mock-phosphorylation reaction. Subsequently, phosphorylated or mock-phosphorylated GST-ASF was incubated with 35 μl of the HeLa cell cytoplasmic extract (equivalent to ~1 ng of proteins) in a 60-μl mixture at 30 °C for 30 min. The reaction mix was then supplemented with an equal volume of NET-2 buffer (50 mM Tris-HCl (pH 7.4), 150 mM NaCl, and 0.05% Nonidet P-40) and subsequently incubated with 10 μl of glutathione-Sepharose at 4 °C for 2 h. The beads were washed extensively with NET-2 buffer. Bound proteins were extracted with SDS lysis buffer and analyzed by immunoblotting with purified anti-SR-SR2 antibodies. The blot was stripped and then reprobed with mAb 104 to examine phosphorylated GST-ASF. To detect both phosphorylated and unphosphorylated GST-ASF, 1/20 volume each of the samples was fractionated in another SDS-polyacrylamide gel electrophoresis and subjected to Western blot analysis using anti-GST antibodies. Western blot analysis was performed by using the enhanced chemiluminescence detection system (Amersham Pharmacia Biotech). To test the interaction of GST-SF with the recombinant TRN-SR2 protein, the pull-down experiments were carried out by using *E. coli* extract containing full-length or ΔN281 TRN-SR2 protein. The reactions were performed as described above. Bound proteins were analyzed as above, or, after blotting with anti-TRN-SR2, the filter was stained with Ponceau S.

For *Ran* competition experiments, reaction mixtures containing...
phosphorylated GST-ASF, TRN-SR2-containing extract, and RanQ69L-GTP or Ran-GDP were incubated at 30 °C for 30 min. Pull-down experiments were performed as described above.

Phosphorylation of the SR peptide [CGGG(RS)8R] was carried out in a 25-μl reaction mix containing 2.5 nmol of the peptide, 50 mM Tris-HCl (pH 7.4), 10 mM MgCl₂, 1 mM dithiothreitol, 0.8 mM ATP, and 0.24 μg of GST-SRPK1 at 30 °C for 45 min. Mock-phosphorylation was carried out in the reaction excluding ATP. Phosphorylated or mock-phosphorylated GST-SRPK1 was added to the reaction mix containing phosphorylated GST-ASF and recombinant TRN-SR2; the pull-down assay was performed as described above.

RESULTS

Nuclear Transport Properties of the HPV-5 E2 Hinge Domain—The E2 protein encoded by epidermodysplasia verruciformis-associated papillomaviruses contains an RS-rich sequence in its hinge region. Previously, we showed that this hinge domain is essential for the colocalization of transiently expressed HPV-5 E2 protein with splicing factors in nuclear speckle domains (6). In an attempt to determine whether the RS-rich hinge is sufficient for nuclear speckle targeting, we inserted the entire hinge or the RS-rich subdomain into a heterologous protein, β-gal, and examined the cellular localization of the fusion protein. Fig. 1 shows that both fusion proteins localized predominantly in the nucleus (panels c and e), whereas β-gal itself was distributed throughout the whole cell (panel a). Although neither of the tested domains was sufficient for speckle targeting, this result suggests that the RS domain of E2 can serve as a functional NLS, like the RS domains or subdomains of some splicing factors (12, 13, 17).

We next asked whether the subcellular localization of the HPV-5 E2 protein could be altered or regulated upon phosphorylation. We first examined the ability of the E2 protein to serve as a substrate for cellular SR protein kinases in vitro. A human SR protein kinase, SRPK1, was transiently coexpressed with the full-length or hinge-deleted E2 protein in transfected HeLa cells. As shown in Fig. 2A, only full-length E2, but not hinge-deleted E2, changed its gel mobility in the presence of overexpressed SRPK1, suggesting phosphorylation of the hinge. Because the E2 protein purified from the baculovirus expression system is readily detectable by mAb 104, which recognizes phosphorylated epitopes in SR proteins (6), E2 might be moderately phosphorylated by a cellular kinase even in the absence of exogenous SRPK1 in HeLa cells. Thus, our finding may indicate that excess SRPK1 extensively phosphorylates the E2 protein in the hinge. Furthermore, the E2 protein, similar to ASF/SF2, appeared to accumulate in the cytoplasm upon hyperphosphorylation of the hinge by excess SRPK1 (Fig. 2B, panels b and f). In contrast, hinge-deleted E2 remained in the nucleus despite the presence of exogenous SRPK1 (panel d). Thus, the hinge of the HPV-5 E2 transactivator behaved similarly to the RS domains of several cellular SR proteins (12, 13, 17) in both its nuclear targeting activity and cytoplasmic distribution upon hyperphosphorylation.

Interaction of Human TRN-SR2 with the E2 Hinge Domain—We next performed a yeast two-hybrid screen to search for HPV-5 E2-interacting proteins from a HeLa cell cDNA library. We wished to identify candidates that could specifically recognize the RS domain of E2 and function as a nuclear transporter. A screen of \(3.5 \times 10^9\) primary transformants yielded 37 positive clones. The majority of the isolated clones encoded known proteins including SF2p32, ASF/SF2, SC35, 9G8, Tra2β, and ribosomal proteins S4 and S14 (data not shown). One partial cDNA encoded a protein of about 400
amino acid residues homologous to the C terminus of yeast importin-β family protein Mtr10p and almost identical to human transportin-SR (Ref. 37; hereafter termed TRN-SR1) and thus attracted our further attention. Such a clone had no detectable interaction with hinge-deleted E2, suggesting that its encoded protein interacts only with the RS-rich hinge domain of E2 (data not shown). Therefore, it is named transportin-SR2 (abbreviated as TRN-SR2).

Northern blot analysis with the 3' end of the coding region of human TRN-SR2 revealed a single transcript of ~4.5 kilobase pairs that is ubiquitously expressed in all human tissues, with higher abundance in testis (data not shown). A ~3.4-kilobase pair cDNA containing the possible entire open reading frame of human TRN-SR2 was obtained by screening a ZAP cDNA library made from HeLa cells. The human TRN-SR2 protein of 923 amino acid residues is ~23% identical to S. cerevisiae Mtr10p and also shares similarities with the putative homologs in several other species (Fig. 3, bottom panel). Intriguingly, TRN-SR2 lacks two regions of ~30 amino acid residues from TRN-SR1 (37) as shown in Fig. 3 (top panel). Further experiments are needed to clarify the relationship between these two proteins. In addition, human TRN-SR2 exhibited significant homology (~22%) to another human open reading frame, namely the KIAA0724 protein (38), throughout the entire sequence. However, this putative importin-β family protein did not interact with SR proteins, as judged by the yeast two-hybrid interaction assay (data not shown).

Interaction of TRN-SR2 with SR Proteins—Since E2’s RS-rich hinge behaved similarly to some splicing factors’ RS domains in many different aspects (6 and see above), we next tested the interaction of TRN-SR2 with cellular SR splicing factors by using the yeast two-hybrid interaction assay. As shown in Fig. 4, human TRN-SR2 interacted with three tested SR proteins, i.e. ASF/SF2, SC35, and Tra2β, via its C-terminal 400 amino acid residues. TRN-SR2 interaction was also detected with truncated Tra2β, which possessed only the N-terminal RS domain, but not with the RNA binding domains of ASF and Tra2β (Fig. 4). This result suggests that the RS domain is sufficient to mediate the interaction of SR proteins with TRN-SR2. Thus, TRN-SR2 can probably interact with the whole family of SR protein splicing factors. Since cellular SR proteins represent key regulators in the expression of eukaryotic genes, and moreover, the hinge itself is dispensable for the nuclear entry of the E2 transactivators (6, 39), we focused further experiments on SR splicing factors instead of HPV E2.

To test whether phosphorylation plays any role in TRN-SR2-SR protein interactions, we performed a protein-protein interaction assay in a SR protein kinase-deficient (sky1Δ) yeast strain (31). The interaction of TRN-SR2 with either ASF/SF2 or SC35 was severely affected by the sky1Δ mutation (Table I). Expression of human SRPK1 in sky1Δ yeast not only rescued such interactions but also increased the apparent binding affinity of TRN-SR2 for SR proteins (Table I).

The table below summarizes the interactions of TRN-SR2 with SR proteins.

| Preys       | β-gal activity |
|-------------|---------------|
| ASF         | 645.0 ± 65.0  |
| ASF-RRM     | 1.1 ± 1.0     |
| Tra2β       | 201.0 ± 49.6  |
| Tra2β-RS1   | 185.7 ± 23.0  |
| TRN-SR2     | 0.8 ± 0.2     |
| SC35        | 147.3 ± 39.9  |

![Fig. 4. TRN-SR2-SR protein interactions in the yeast two-hybrid system. A pair of plasmids expressing Gal4 AD-TRN-SR2 (bait) and one of the LexA DB-SR protein fusions (prey; listed at the left), respectively, were co-transformed into the LacZ reporter-containing yeast strain EGY48. Quantitative liquid β-galactosidase assay was performed, and data are expressed as β-gal activity (units) averaged from at least five independent isolates for each combination. A β-gal unit is defined as the amount of β-galactosidase that hydrolyzes 1 μmol of ONPG/min/cell. Combinations of the empty bait and various prey gave rise to very low β-gal activities (<1) (data not shown). Schematics of SR proteins and their domains are presented; filled boxes represent RS domain.](http://www.jbc.org/)
Phosphorylation of the RS domains influences the interaction of SR proteins with TRN-SR2 in the yeast two-hybrid system.

Pairs of bait (Gal4 AD fusions) and prey (LexA DB fusions) plasmids were co-transformed into LacI reporter containing wild-type yeast strain EGY48 (SKY1), or SKY1 expressing SRPK1. Quantitative liquid β-galactosidase assays were performed on at least five independent isolates for each combination. Results are expressed as percentage of averaged β-gal activity of each strain compared to that of wild type.

| Strain | TRN-SR2: ASF | TRN-SR2: SC35 | PRP19: SNT309 |
|--------|--------------|--------------|---------------|
| SKY1   | 100          | 100          | 100           |
| sby1Δ  | 10.8         | 3.7          | 100           |
| sby1Δ + SRPK1 | 141         | 128          | 93            |

in sby1Δ yeast in part resulted from inefficient nuclear localization of unphosphorylated SR proteins (31). Therefore, TRN-SR2-SR protein interactions were then examined by using in vitro biochemical approaches (see below).

In Vitro Interaction of TRN-SR2 with a Phosphorylated SR Protein—To examine whether TRN-SR2 can interact with SR proteins in vitro, we used a GST-ASF fusion protein to perform a pull-down assay in the HeLa cell cytoplasmic extract. GST-ASF was overproduced in bacteria where it should not be modified by phosphorylation. Purified GST-ASF was then phosphorylated by SRPK1 in vitro and thus became detectable by mAb 104 (Fig. 5A, lane 2). Interestingly, only phosphorylated, but not unphosphorylated, GST-ASF specifically selected TRN-SR2 from the HeLa cell extract, suggesting that TRN-SR2 or its associated complex can recognize only phosphorylated ASF in vitro (lanes 4 and 5). Western blotting with anti-GST antibodies excluded the possibility that unphosphorylated GST-ASF was degraded during its incubation with the cell extract (lane 5).

It is well known that an elevated concentration of Ran-GTP in the nucleus can trigger the dissociation of cargo from the import complex (18–21). We therefore tested whether RanQ69L-GTP, which is resistant to activation of its GTPase activity by cytosolic RanGAP, could interfere with the binding of ASF to TRN-SR2 in the HeLa cell extract. As shown in Fig. 5B, phosphorylated GST-ASF failed to select TRN-SR2 from the extract in the presence of RanQ69L-GTP (lanes 2 and 3), whereas Ran-GDP had no effect on their interaction (lanes 4 and 5). Thus, TRN-SR2 meets the criteria for being an import factor, probably for SR proteins.

TRN-SR2 Directly Binds to a Phosphorylated RS Motif—Since neither of the above experiments could exclude the possibility that the interactions between TRN-SR2 and SR proteins were via a yeast or other HeLa cell protein (for example, importin-α or its analog), recombinant TRN-SR2 was expressed in E. coli and used in an in vitro protein-protein interaction assay. As shown in Fig. 6A, TRN-SR2 in the bacterial extract can bind to phosphorylated, but not unphosphorylated, GST-ASF (lanes 2 and 3), consistent with the above result. Interestingly, TRN-SR2 can also bind efficiently to ASF that is thio-phosphorylated by ATP-γS (lane 4), confirming the importance of phosphate moieties of the RS domain in TRN-SR2 interaction. Next, a synthetic peptide containing eight consecutive RS dipeptide repeats was used to test whether it is sufficient for binding to TRN-SR2 in a competition experiment. The SR peptide was phosphorylated or mock-phosphorylated by SRPK1; on average, two serines became phosphorylated in a single molecule of the peptide (data not shown). As shown in Fig. 6B, only the phosphorylated SR peptide competed for the binding of TRN-SR2 to GST-ASF (lanes 5 and 6). Thus, all of the above data are consistent with the idea that TRN-SR2 can specifically recognize phosphorylated RS domains.

A Truncated TRN-SR2 Mutant Accumulates in the Speckled Domains of HeLa Cell Nuclei—We next examined the cellular localization of transiently expressed TRN-SR2 protein by indirect immunofluorescence. HeLa cells were transfected with a vector that expresses HA epitope-tagged TRN-SR2 and, immunofluorescence staining was performed using purified anti-TRN-SR2 antibodies or anti-HA antibody. Full-length TRN-SR2 in transfected cells was stained throughout the whole cell using either of the antibodies (Fig. 7A, panels a and b). Anti-TRN-SR2 antibodies detected very faint signals corresponding to the endogenous TRN-SR2 protein in mock-transfected cells (data not shown). Surprisingly, a truncated form of TRN-SR2, ∆N281, localized predominantly in the nucleoplasm with additional concentration in nuclear punctate domains (panels c and d), reminiscent of the distribution pattern of splicing factors. Accordingly, when cells were treated with 5,6-dichloro-1-bromo-3-(3-dimethylaminonaphthalene)-2(1H)-one (DBR) (see Ref. 10 and references therein), the speckled pattern produced by the anti-HA antibody changed significantly, i.e. round and bright in fluorescence intensity (panels e and f). This result indicates the redistribution of AN281 by inhibition of RNA pol II transcription, as observed with SR proteins (10). However, DBR treatment had no effect on the staining pattern of full-length TRN-SR2 that was transiently expressed in transfected cells (data not shown). We next performed a colocalization experiment in DRB-treated cells, since the punctate signals of AN281 in untreated cells were too faint to be detected in double staining (data not shown). Strikingly, indirect immunofluorescence using anti-HA and anti-SC35 antibodies revealed that the speckled staining patterns of AN281 overlapped well with those of SC35 in DRB-treated cells (panels g–i).

![Fig. 5. In vitro interaction of phosphorylated GST-ASF with TRN-SR2 in the HeLa cell extract.](image-url)
The above results suggested that the truncated TRN-SR2 protein may so tightly interact with SR proteins in the nucleus that GTP-bound Ran would not have any effect. To test this possibility, an in vitro pull-down experiment was performed. In contrast to full-length TRN-SR2, the binding of phosphorylated GST-ASF to DN281 was not competed by the presence of RanQ69L-GTP (Fig. 7B, lanes 3 and 7). Ran-GDP had no effect on the binding of GST-ASF to full-length or DN281 TRN-SR2 (lanes 4 and 8), as expected. Thus, the DN281 mutant might chaperone SR proteins across the nuclear envelope to the speckles, where they accumulate because the interaction is not disrupted by nuclear Ran-GTP. TRN-SR2 is therefore likely to be an import receptor for phosphorylated SR proteins.

DISCUSSION

In the present study, we show that the RS dipeptide-rich hinge of a papillomavirus E2 transactivator serves as an NLS when attached to a heterologous protein. This RS domain is an NLS that differs in its composition from other well-characterized NLSs. It is important to determine whether the pathway for the RS domain-mediated nuclear protein import is also distinct from those used by other classes of NLSs. Our results suggest the involvement of human TRN-SR2 in the cellular trafficking of SR proteins via binding to their phosphorylated RS domain. In addition, TRN-SR1, a protein highly similar to TRN-SR2, was recently reported by Dreyfuss and colleagues (37) to be a functional nuclear import receptor for GST-RS domain fusion proteins in vitro. Thus, RS domain-containing

![A](image1)

**FIG. 6.** In vitro interaction of phosphorylated GST-ASF with recombinant TRN-SR2. Panel A, 2 μg of mock-phosphorylated (lane 2), phosphorylated (lane 3), or thiophosphorylated (lane 4) GST-ASF was incubated with the E. coli extract containing recombinant TRN-SR2 followed by incubation with glutathione-Sepharose. Bound proteins were analyzed by immunoblotting with purified anti-TRN-SR2 antibodies for TRN-SR2, mAb 104 for phosphorylated GST-ASF, and anti-GST antibodies for both phosphorylated and unphosphorylated GST-ASF. Lane 1 represents 1/10 of input E. coli extract. Panel B, phosphorylated GST-ASF was incubated with the E. coli extract containing TRN-SR2 and mock-phosphorylated (lane 3, 0.5 nmol; lane 4, 2.5 nmol) or phosphorylated SR peptide (lane 5, 0.5 nmol; lane 6, 2.5 nmol) or buffer alone (lane 2), followed by incubation with glutathione-Sepharose. Bound TRN-SR2 was detected by immunoblotting using anti-TRN-SR2 and GST-ASF was detected by Ponceau S staining. Lane 1 represents 1/30 of input E. coli extract.

![B](image2)

**FIG. 7.** Cellular localization of full-length and ΔN281 TRN-SR2 in transiently transfected HeLa cells. Panel A, HeLa cells were transiently transfected with vector expressing HA-tagged TRN-SR2 (panels a and b) or HA-tagged ΔN281 (panels c–i). Transfected cells were treated with 100 μM DRB for 4 h before fixation (panels c–i). Indirect immunofluorescence was performed 40 h after transfection using purified anti-TRN-SR2 polyclonal antibodies (panel a), or anti-HA monoclonal antibody (panels b–f), or using anti-HA polyclonal antibodies and anti-SC35 monoclonal antibody for double staining (panels g–l). The secondary antibodies for polyclonal primary antibodies were coupled to rhodamine (red), and those for monoclonal antibodies was coupled to fluorescein (green). Lane 1 represents 1/10 of input E. coli extract.
proteins are likely imported to the nucleus by at least one newly identified import factor in mammalian cells. Moreover, the RS-NLS may consist of alternate charges of residues upon serine phosphorylation. Regulation of the accessibility of the NLS to the transport machinery by phosphorylation is known to be a major mechanism controlling the nucleocytoplasmic transport of proteins (40). Although phosphorylation often occurs at residues adjacent to but not within the regulated NLSs (40), the RS domain, in contrast, contains multiple intrinsic phosphorylation sites that can be directly targeted by SR protein kinases. Phosphorylation indeed alters the nucleocytoplasmic transport properties of some SR proteins (Refs. 17, 41, and 42; this study). This therefore suggests that the RS-NLS may behave as a regulated nuclear import signal (see below).

Our data show that the E2 transactivator can be extensively phosphorylated and accumulate in the cytoplasm in the presence of excess SRPK1. Such a phenomenon was also observed with ASF, a shuttling SR protein (17, 41). Thus, it was proposed that hyperphosphorylation of the RS domain can either accelerate the nuclear export of shuttling SR proteins or block their reimport (17). However, the E2 transactivator, unlike ASF, is incapable of shuttling between cytoplasm and nucleus.2 (unpublished data). Therefore, nuclear import rather than export of E2 and probably other SR proteins may be impeded by exogenous SRPK1. Although phosphorylation of the RS domain is important for efficient nuclear entry of SR proteins (31), extensive phosphorylation of the RS domain in the cytosol appears to have an opposite effect in SR protein trafficking (Refs. 17, 41, and 42; this study). However, at present, a possibility that still remains to be investigated is whether hyperphosphorylation and cytoplasmic accumulation of SR proteins are independent consequences of the presence of excess SRPK1. Moreover, an important question is whether overexpression of SR protein kinases is biologically relevant. In fact, the kinase activity of SRPK1 increases significantly during mitosis and, accordingly, SR proteins redistribute (14). Thus, EV-HPV E2 transactivators might relocate, probably coordinately with SR proteins, during mitosis via hyperphosphorylation of the hinge.

Our results indicate that phosphorylation of the RS domain is critical for the interaction of ASF and possibly other SR proteins with TRN-SR2. In yeast, nuclear import of SC35, of which the RS domain serves as the only NLS, was severely impeded when the SR protein kinase was deleted (31). Thus, unphosphorylated SC35 apparently fails to interact with its corresponding import factor in yeast and accumulates in the cytoplasm. While it remains uncertain whether nuclear import of SC35 is mediated by Mtr10p in yeast, our data indicating that human TRN-SR2 has a stronger preference for phosphorylated RS domains are consistent with such a hypothesis. In contrast, it appears that TRN-SR1 can recognize unphosphorylated SR proteins as import substrates or that TRN-SR1 does not discriminate between phosphorylated and unphosphorylated SR proteins (37). Thus, whether the two proteins exhibit distinct specificity to differently phosphorylated SR proteins needs to be clarified in the future. At present, the extent of SR domain phosphorylation required to achieve SR proteins’ optimal interaction affinity with TRN-SR2 remains unclear. A SR peptide containing two phosphoserines (in average) was enough for TRN-SR2 binding (Fig. 6); still, questions remain to be answered as to which serine residues are phosphorylated in optimal substrates and whether full phosphorylation of the RS domain would decrease its affinity for TRN-SR2. The structural features of TRN-SR2 in complexes with phosphorylated SR proteins remain to be determined through future studies.

In a competition experiment, we observed that the SR peptide had lower affinity than ASF for TRN-SR2 (Fig. 6). Analogously, a synthetic peptide derived from the NES of the human immunodeficiency virus Rev protein was shown to have much lower affinity in binding to CRM1 when compared with the entire Rev protein (43). It is possible that the conformation of an isolated peptide is different from that within a native protein, or that the residues outside the RS repeats or Rev NES stabilize the interactions with corresponding importin-β proteins. Previously, transfection experiments showed that the Drosophila Tra protein contains several nuclear and subnuclear localization signals (12). Those signals are composed of consecutive RS dipetide repeats and several basic amino acid residues directly adjacent to the RS stretches (12). In the case of the importin-α IBB domain, the extended N-terminal moiety is important for stabilizing the interaction of the IBB helix with importin-β (29, 44). Thus, it will be interesting to test whether the basic residues could cooperate with their adjacent RS repeats in binding to transportin-SRs. On the other hand, the stretch of the basic residues in the Tra RS domain was implicated to function as a nucleoplasm-in-like bipartite NLS (12). Therefore, it is also possible that different mechanisms exist for RS domain-mediated nuclear import.

An N-terminally deleted TRN-SR2 mutant (∆N281) was predominantly located in the nucleus and, most interestingly, concentrated in the speckles where SR proteins locate. We reasoned that the ∆N281 mutant might be recruited to the nuclear speckles via its binding to SR proteins and that the interaction of ∆N281 with SR proteins was not disturbed by Ran-GTP in the nucleus. In addition, recycling of TRN-SR2 to the cytoplasm may also be hampered by removing its N-terminal Ran-interacting domain. However, the speckle localization pattern was not observed with the full-length TRN-SR2 protein (data not shown) nor with transportin and its truncated versions (45). In conclusion, our results provide in vivo evidence to suggest that TRN-SR2 might play a role in escorting at least some SR proteins or speckle-localized proteins in cellular trafficking. Interestingly, at least a portion of the ∆N281 mutant form of TRN-SR2 might be tightly associated with SR proteins such that this portion of the ∆N281 protein distributed coordinately with SR proteins between the subnuclear domains, particularly upon the change in the states of RNA pol II transcription. Whether such a TRN-SR2 mutant confers a dominant-negative effect in the nucleocytoplasmic transport of SR proteins or even in their splicing functions remains further examination.

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REFERENCES

1. Fu, X. D. (1995) RNA 1, 663–680.
2. Manley, J. L., and Tacke, R. (1996) Genes Dev. 10, 1569–1579.
3. Valcarcel, J., and Green, M. R. (1996) Trends Biochem. Sci. 21, 296–301.
4. Zahler, A. M., Lane, W. S., Stolk, J. A., and Roth, M. B. (1992) Genes Dev. 6, 847.
5. Birney, E., Kumar, S., and Kraainer, A. R. (1993) Nucleic Acids Res. 21, 5803–5816.
6. Lai, M.-C., Teh, B. H., and Tarn, W.-Y. (1999) J. Biol. Chem. 274, 11832–11841.
7. Yuriev, A., Patturajan, M., Litintung, Y., Joshi, R. B., Gentile, C., Gebarsa, M., and Corden, J. L. (1996) Proc. Natl. Acad. Sci. U. S. A. 93, 6975–6980.
8. Puigserver, P., Wu, Z., Park, C. W., Graves, R., Wright, M., Spiegelman, B. M. (1998) Cell 92, 829–839.
9. Ruegsegger, U., Blank, D., and Keller, W. (1998) Mol. Cell 1, 243–253.
10. Spector, D. (1996) Exp. Cell Res. 229, 189–197.
11. Singer, R. H., and Green, M. R. (1997) Cell 91, 291–294.

2 M.-C. Lai and W.-Y. Tarn, unpublished data.
Transportin-SR2 Interacts with the Phosphorylated RS Domain

12. Hedley, M. L., Amrein, H., and Maniatis, T. (1995) Proc. Natl. Acad. Sci. U. S. A. 92, 11524–11528
13. Caceres, J. F., Mistelli, T., Sreeraman, G. R., Spector, D. L., and Krainer, A. R. (1997) J. Cell Biol. 138, 225–238
14. Gui, J. F., Lane, W. S., Fu, X. D. (1997) J. Cell Biol. 138, 225–238
15. Caceres, J. F., Mistelli, T., Sreeraman, G. R., Spector, D. L., and Krainer, A. R. (1998) Genes Dev. 12, 55–66
16. Nigg, E. A. (1997) Nature 386, 779–787
17. Gorlich, D. (1998) EMBO J. 17, 2721–2727
18. Izaurralde, E., and Adam, S. (1998) RNA 4, 351–364
19. Mattaj, I. W., and Englmeier, L. (1998) Annu. Rev. Biochem. 67, 265–306
20. Michael, W. M., Choi, M., and Dreyfuss, G. (1995) Cell 83, 415–422
21. Siomi, H., and Dreyfuss, G. (1995) J. Cell Biol. 129, 551–560
22. Palmieri, D., and Malim, M. H. (1999) Mol. Cell. Biol. 19, 1218–1222
23. Pemberton, L. F., Rosenblum, J. S., and Blobel, G. (1997) J. Cell Biol. 139, 1645–1653
24. Chook, Y. M., and Blobel, G. (1999) Nature 399, 230–237
25. Cingolani, G., Petosa, C., Weis, K., and Muller, C. W. (1999) Nature 399, 221–229
26. Mattaj, I. W., and Conti, E. (1999) Nature 399, 208–310
27. Yeakley, J. M., Tronchere, H., Olesen, J., Dyck, J. A., Wang, H. Y., and Fu, X. D. (1999) J. Cell Biol. 145, 447–455
28. Chen, H.-R., Jan, S.-P., Tsao, T. Y., Sheu, Y.-J., Banroques, J., and Cheng, S.-C. (1998) Mol. Cell. Biol. 18, 2196–2204
29. Gorlich, D., Pante, N., Kutay, U., Aebl, U., and Bischoff, F. R. (1996) EMBO J. 15, 5984–5994
30. Floer, M., and Blobel, G. (1999) J. Biol. Chem. 274, 16279–16286
31. Adam, S. A., Sterne-Marr, R., and Gerace, L. (1991) J. Cell Biol. 111, 807–816
32. Paschal, B. M. (1998) in Cell Biology: A Laboratory Handbook (Celis, J. E., eds) Vol. 2, pp. 305–313, Academic Press, Orlando, FL
33. Kataoka, N., Bachorik, J. L., and Dreyfuss, G. (1999) J. Cell Biol. 145, 1145–1152
34. Nagase, T., Ishikawa, K., Suyama, M., Kikuno, R., Miyajima, N., Tanaka, A., Kotani, H., Nomura, N., and Ohara, O. (1998) DNA Res. 5, 277–286
35. Sknadopoulos, A. H., and McBride, A. A. (1996) J. Virol. 70, 1117–1124
36. Jans, D. A., Chan, C. K., and Huber, S. (1996) Physiol. Rev. 76, 651–685
37. Koizumi, J., Okamoto, Y., Onogi, H., Miyeda, A., Krainer, A. R., and Hagiwara, M. (1999) J. Biol. Chem. 274, 11125–11131
38. Kuroyanagi, N., Onogi, H., Wakabayashi, T., and Hagiwara, M. (1998) Biochem. Biophys. Res. Commun. 242, 357–364
39. Paraskeva, E., Izaurralde, E., Bischoff, F. R., Huber, J., Kutay, U., Hartmann, E., Luhrmann, R., and Gerlich, D. (1999) J. Cell Biol. 145, 255–264
40. Weis, K., Mattaj, I. W., and Lamond, A. I. (1999) Science 288, 1049–1053
41. Nakielny, S., and Dreyfuss, G. (1998) Curr. Biol. 8, 89–95
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