

Transportin2 functions as importin and mediates nuclear import of HuR

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The RanGTP-binding nuclear transport receptors transportin1 (TRN1) and transportin2 (TRN2) are highly similar in sequence but are reported to function in nuclear import and export, respectively. Here we show that TRN2 possesses properties of a nuclear import receptor. TRN1/2 both interacted with a similar set of RNA-binding proteins in a RanGTP-sensitive manner. TRN2 bound RanGTP with high affinity, a feature of nuclear import receptors. As expected of an import complex, RanGTP also disrupted the interaction between TRN2 and HuR, an RNA-binding protein previously described as a TRN2 export substrate. The HuR nucleocytoplasmic shuttling signal, a sequence resembling the M9 nuclear import signal of hnRNP A1, was necessary and sufficient for TRN-mediated nuclear import of HuR *in vitro*. Finally, crosscompetition experiments demonstrated that HuR nucleocytoplasmic shuttling signal and M9 are imported along redundant pathways involving TRN1/2, substantiating the function of TRN2 in nuclear import.

The exchange of molecules between the nucleus and cytoplasm occurs through nuclear pore complexes (NPC). Proteins and RNAs are carried through the NPC by way of transport receptors that recognize specific signals on their cargo molecules. The importin β /karyopherin family of transport receptors mediates the majority of nuclear import and export processes. These importins and exportins interact with the GTP-bound form of the GTPase Ran to control cargo binding and release in a compartment specific manner. Importins require an environment free of RanGTP to bind their cargo, a condition satisfied in the cytoplasm, and they release their cargo in the nucleus where RanGTP is concentrated. Conversely, exportins require nuclear RanGTP to bind their cargo, and they dissociate from their cargo in the cytoplasm where RanGTP is converted to RanGDP (for review, see refs. 1 and 2).

Although the majority of nucleocytoplasmic exchange relies on RanGTP-responsive transport receptors, Ran-independent pathways also exist. One example is nuclear export of mRNAs, which utilizes the RanGTP-insensitive transport receptor heterodimer TAP/p15 (Mex67p/Mtr2p in yeast) (for review, see refs. 3–6). TAP and Mex67p are members of the evolutionarily conserved NXF family, which shares with exportins the property of binding directly to the NPC (7).

Although most cellular mRNAs are exported by NXF family members (8), two RanGTP-binding transport receptors, CRM1 and transportin2 (TRN2, also referred to as karyopherin β 2b), have been suggested to contribute to the export of specific mRNAs in higher eukaryotes. CRM1, for example, appears to export yet unidentified mRNAs that use NXF3, a TAP variant lacking an NPC-binding domain (9). CRM1 further supports nuclear export of specific viral mRNAs (for review, see ref. 10). Finally, the export of specific cellular mRNAs, like *c-fos*, depends on functional CRM1 (11).

c-fos mRNA belongs to a group of mRNAs that contain AU-rich elements (AREs) in their 3' untranslated region. AREs are found in many short-lived cytokine and protooncogene mRNAs, and they regulate mRNA half-life through interaction with several proteins. One of these ARE-binding proteins is HuR (12). HuR not only has a stabilizing effect on ARE-containing mRNAs but has also been

proposed to function as an adaptor protein recruiting export receptors to the *c-fos* message (13).

Two transport receptors, CRM1 and TRN2, have been implicated in the nucleocytoplasmic transport of HuR and *c-fos* mRNA. CRM1 is recruited to HuR/mRNA complexes by two additional factors, APRIL and pp32. TRN2 also interacts with HuR in cell lysates but a direct interaction with HuR or HuR/mRNA complexes has not yet been demonstrated. Several lines of evidence suggest that HuR is involved in *c-fos* mRNA export in conjunction with CRM1 and TRN2 (1). HuR is a nucleocytoplasmic shuttling protein (2, 14–16). Leptomycin B, a drug that inactivates CRM1, partially blocks *c-fos* mRNA export (3, 11). Cell-permeable peptides that compete for transport substrate binding to CRM1 or to TRN2 block not only HuR shuttling but also *c-fos* mRNA export (13).

TRN2 exists in two isoforms, both highly similar to the importin TRN1, which functions in nuclear import of heterogeneous nuclear ribonucleoproteins (hnRNP) like hnRNP A1 (17–20). The two TRN2 isoforms can be distinguished by a 10-aa insertion in the C-terminal part of the molecule, presumably generated by alternative splicing. Despite the high degree of sequence resemblance between the importin TRN1 and the two TRN2 variants, both forms of TRN2 were proposed to function as export receptors. The long TRN2 variant was implicated in nuclear export of HuR (13), and the short form of TRN2 was reported to participate in general poly(A)⁺ mRNA nuclear export by way of a RanGTP-dependent interaction with the mRNA export receptor TAP (20). In this study, we set out to identify additional binding partners of TRN2 and to determine how TRN1/2 differ in their RanGTP-controlled association with cargo molecules. Unexpectedly, we found that TRN1/2 possess identical properties characteristic of nuclear import receptors.

Materials and Methods

Molecular Cloning. The coding region of TRN2 was amplified by PCR by using HeLa cell cDNA as a template. The PCR fragments were cloned into the *NcoI*-*Bam*HI sites of pQE60–2z or into the *Bam*HI/*Xma*I sites of pQE30 (Qiagen, Chatsworth, CA). Likewise, the coding regions of human HuR, its various deletion derivatives (Fig. 6A, which is published as supporting information on the PNAS web site), polyglutamine tract-binding protein (PQBP-1) and the M9 domain, were amplified by PCR from HeLa cell cDNA and cloned into the *NcoI*/*Bam*HI sites of pQE60–6z (21) or –2z.

Parts of the 3' untranslated region of vascular endothelial growth factor (VEGF) were amplified from human keratinocyte cDNA (kind gift of S. Werner, Eidgenössische Technische Hochschule, Zürich) by PCR by using the primers VEGF 260 sense (GCCGGG-TACCTTATGTATATATGTGATTCTGATAAAATAGAC) and VEGF 260 antisense (GGCCAAGCTTCGTTGTTTAAAA-ATAAATATGTACTACGG) and cloned into the *KpnI*/*Hind*III

Abbreviations: TRN, transportin; HNS, HuR nucleocytoplasmic shuttling signal; NPC, nuclear pore complex; ARE, AU-rich element; hnRNP, heterogeneous nuclear ribonucleoprotein; VEGF, vascular endothelial growth factor.

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sites of pBSKII(+). VEGF-260 (263 nt in length) starts 1,601 nt downstream of the VEGF stop codon; VEGF-110 (110 nt) starts at the same position.

Recombinant Protein Expression. The expression and purification of TRN1, Imp β , - α , -5, -7, -9, RanQ69L, GST-M9, and IBB-GST have been described (22).

2z-PQBP-1, 6z-PQBP-1, 2z-HuR, 6z-M9, 6z-HuR, and other zz-tagged HuR derivatives were expressed in BLR(pREP4) at 25°C by induction with 1 mM isopropyl β -D-thiogalactoside. Cells were lysed by sonication in 50 mM Tris, pH 7.5/200 mM NaCl/5 mM MgCl₂/5% glycerol/2 mM 2-mercaptoethanol. The lysate was cleared by ultracentrifugation, then passed over Ni-NTA agarose (Qiagen) and eluted with 400 mM imidazole in lysis buffer. Peak fractions were pooled and the buffer exchanged to 50 mM Tris, pH 7.5/200 mM K acetate/2 mM MgCl₂.

2z-TRN1/2 and N-His TRN2 were expressed in XL1(pBS161) at 21°C. Cells were lysed by sonication in 50 mM Tris, pH 7.5/700 mM NaCl/10 mM MgCl₂/5% glycerol/5 mM 2-mercaptoethanol. Purification was performed by chromatography on Ni-NTA agarose, followed by MonoQ and gel filtration in 50 mM Tris, pH 7.5/100 mM K acetate/1 mM Mg acetate.

Protein Identification by MS. After affinity chromatography on 2z-TRN1/2, bound proteins were eluted, separated by SDS/PAGE, and stained with Coomassie blue. The protein bands of interest were excised from the gel and subjected to tryptic digestion. The tryptic peptides were analyzed by LC-tandem MS (LCQ Deca XP, Finnigan-MAT, San Jose, CA) and database search performed by using SEQUEST in a human National Center for Biotechnology Information protein database.

Solution-Binding Experiments. Pull-down from HeLa cell extracts. HeLa cell extract was prepared as described in ref. 23. In Fig. 1, for each reaction, 1.5 ml of HeLa cell extract (4 mg/ml in 50 mM Tris, pH 7.5/150 mM K acetate/5 mM Mg acetate) was incubated with 1 μ g/ml latrunculin B and, where indicated, with 0.1 μ g/ μ l RNase A, for 20 min at 37°C. Then, purified 2z-TRN1/2 (1 μ M each) and RanQ69L(GTP) (5 μ M) were added and incubated further for 4 h on ice. After centrifugation for 10 min at 16,000 \times g at 4°C, the supernatant was mixed with 20 μ l of IgG-Sepharose beads for 1 h. Beads were washed three times in binding buffer. Bound proteins were eluted with 1.5 M MgCl₂/50 mM Tris, pH 7.5, precipitated with isopropanol, and dissolved in SDS sample buffer.

For Fig. 2 and Fig. 7, which is published as supporting information on the PNAS web site, 2z-HuR-tagged proteins were preimmobilized on 20 μ l of IgG-Sepharose to a concentration of 2–4 mg/ml Sepharose beads. Beads were incubated with 0.4 ml of HeLa cell extract (7 mg/ml) in either binding buffer A (Fig. 2, 50 mM Tris, pH 7.5/125 mM NaCl/75 mM K acetate/2 mM MgCl₂) or binding buffer B (Fig. 7, 50 mM Tris, pH 7.5/200 mM K acetate/2 mM MgCl₂) for 4 h at 4°C. RanQ69L(GTP) was added to 5 μ M. After washing in the respective binding buffer, bound proteins were eluted as described above.

Pull-down from Escherichia coli lysates. For Figs. 3A and 8B, purified full-length 6z-HuR or 6z-tagged HuR fragments were preimmobilized on 20 μ l of IgG-Sepharose to a concentration of 2–4 mg of protein per ml of IgG-Sepharose beads. *E. coli* lysates (in 50 mM Tris, pH 7.5/150 mM K acetate/2 mM MgCl₂) were supplemented with purified TRN1/2 (2 μ M each) and incubated with the beads for 3 h at 4°C. After washing in the respective binding buffer, bound proteins were eluted as described above.

Dissociation of HuR/TRN Complexes. 6z-HuR was preimmobilized on 105 μ l of IgG-Sepharose beads to saturation. Each 50 μ l of beads was incubated with 720 pmol of TRN1/2 in 50 mM Tris, pH 7.5/200 mM K acetate/2 mM MgCl₂ for 1 h at 4°C. Beads were washed and each sample split into three equal parts

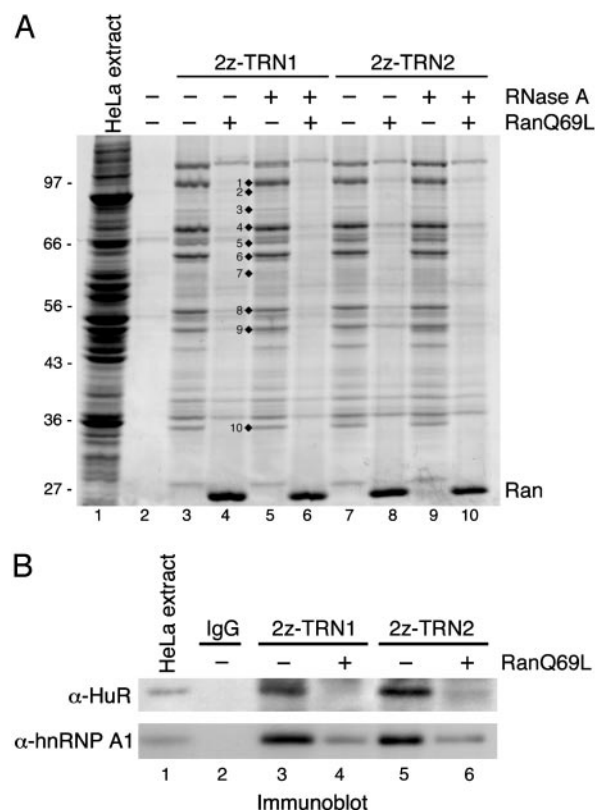


Fig. 1. Identification of TRN1/2-interacting proteins. (A) HeLa cell extract was incubated with 2z-TRN1/2 in the absence or presence of RanQ69L. TRN1/2-associated proteins were retrieved on IgG-Sepharose. Binding to IgG beads (lane 2) served as negative control. Where indicated, the HeLa extract had been pretreated with RNase A. Bound proteins were eluted, separated by SDS/PAGE, and detected by Coomassie blue staining. Protein bands indicated by numbers were subjected to MS. Load in the input lane equals 1/60 of the load in the bound fractions. (B) 2z-TRN1/2-bound fractions were subjected to immunoblotting with antibodies to HuR or hnRNP A1.

containing 15 μ l of beads. One part was directly eluted with SDS sample buffer. The remaining samples were incubated with either 100 μ l of binding buffer or buffer supplemented with RanQ69L(GTP) (10 μ M). After 10 min at room temperature, eluted protein was separated from the beads, precipitated with isopropanol, and analyzed by SDS/PAGE.

Binding of VEGF-260/-110 RNA to 2z-HuR. *In vitro* transcribed VEGF-260 RNA (400 pmol, 35 μ g) or VEGF-110 RNA (960 pmol, 35 μ g) was incubated with 2z-HuR (220 pmol) for 20 min at 4°C in binding buffer [50 mM Tris, pH 7.5/200 mM NaCl/2 mM MgCl₂/0.25 μ g/ μ l BSA/1.5 ng/ μ l poly(A) RNA (Sigma), sample volume 200 μ l]. After centrifugation for 10 min at 16,000 \times g at 4°C, the supernatant was mixed with 10 μ l of IgG-Sepharose beads for 10 min to retrieve 2z-HuR/RNA complexes. Beads were washed two times in binding buffer and once in 50 mM Tris, pH 7.5. HuR-bound RNA was released from the beads by proteinase K digestion followed by phenol extraction and ethanol precipitation.

Antibodies. Anti-Imp β , -5, and -7 were kind gifts of D. Görlich (Zentrum für Molekulare Biologie der Universität Heidelberg, Heidelberg). Antibodies against Exp5, CRM1 have been described (24). Antibodies against TRN1/2 were raised in rabbits by using peptides C-SDQFPLPLKERLAIFYGV (TRN1) as antigen. Affinity purification was performed by using sulfoLink. Note that the anti-TRN1 antibody efficiently crossreacts with

Table 1. List of TRN1/2-interacting proteins as identified by tandem MS

No.	M_r	Proteins	GenBank accession no.	TRN1	TRN2
1	90	RNA-binding protein EWS	Q01844	+	n.a.
		HSP90	P07900	+	n.a.
2	85	Cyclin T1	O60563	+	n.a.
3	74	RNA helicase DDX3	O00571	+	n.a.
4	68	hnRNP M	P52272	+	+
		RNA-binding protein FUS	S33799	+	+
		HSP70	P11142	+	+
5	66	Pre-mRNA cleavage factor I 68-kDa subunit (CPSF6)	NP_008938	n.a.	+
6	60	GAP-associated tyrosine phosphoprotein p62 (Sam68)	A38219	+	+
		TCP-1- γ	P49368	+	+
7	58	HMBA-inducible protein	AAH06460	+	+
8	53	hnRNP H1	P31943	+	+
9	49	hnRNP F	S43484	+	n.a.
		Y box-binding protein 1	I39382	+	n.a.
10	34	hnRNP A1	S04617	+	n.a.
		PQBP-1 (polyglutamine tract-binding protein)	NP_005701	+	n.a.

n.a., not analyzed.

TRN2 as tested by immunoblotting on the recombinant proteins (not shown). The anti-HuR monoclonal antibody 3A2 was purchased from Santa Cruz Biotechnology, and the anti-hnRNP A1 antibody was a kind gift of M. Carmo-Fonseca (University of Lisbon, Lisbon).

RanGTPase Assay. The RanGTPase assay was performed by using the charcoal method as described (25).

In Vitro Nuclear Import Assay. *In vitro* transport reactions were performed essentially as described (21, 26). Import substrates were labeled with stoichiometric amounts of Alexa 488 (Molecular Probes) for 30 min on ice. Unincorporated dye was removed by buffer exchange over NAP5 columns (Pharmacia).

HeLa cells were grown on coverslips and permeabilized with digitonin (40 $\mu\text{g}/\text{ml}$). All import reactions, unless indicated otherwise, contained an energy regenerating system and Ran mix. The import buffer contained 20 mM Hepes, pH 7.5/140 mM K acetate/2 mM Mg acetate/0.5 mM EGTA/250 mM sucrose/2 $\mu\text{g}/\mu\text{l}$ nucleoplasmin core. Import active HeLa cell extract (7.5 mg/ml) was produced by hypotonic lysis of HeLa cells by using 5 mM Tris, pH 7.5/1 mM Mg acetate/0.5 mM EGTA and subsequent adjustment to 20 mM Hepes, pH 7.5/110 mM K acetate/2 mM Mg acetate/0.5 mM EGTA/250 mM sucrose. After 15 min of import, cells were fixed with 3.7% paraformaldehyde.

In Vitro Transcription. VEGF-110 and -260 RNAs were generated by *in vitro* transcription by using T7 RNA polymerase [Ambion (Austin, TX) MEGashortscript Kit] and a PCR product or *Hind*III linearized plasmid DNA as templates, respectively. Unincorporated nucleotides were removed by passage over Sephadex G-50 columns (Pharmacia). RNAs were phenol extracted, precipitated with ethanol, and solved in water.

Electrophoretic Mobility-Shift Assay (EMSA). Purified proteins and RNAs [2z-HuR (1.75 μM), TRN2 (1.5 μM), RanQ69L (10 μM), VEGF-260 (7 μg), and VEGF-110 (7 μg)] were incubated in EMSA buffer (10 mM Tris, pH 7.5/80 mM KCl/0.5 mM Mg acetate/0.5 mM DTT/10% glycerol; sample volume 20 μl) in the presence of 100 pmol unspecific competitor RNA (U6 Δ ss) for 10 min on ice. Samples were loaded on 6% nondenaturing polyacrylamide gels and run at a constant voltage of 10 V cm^{-1} in 0.5 \times TBE (45 mM

Tris base/45 mM boric acid/1.25 mM EDTA, pH 8.3) at room temperature. Gels were stained with Coomassie blue.

Results

Two isoforms of TRN2 have been reported that differ by a 10-aa insertion in the C-terminal part of the molecule that breaks the otherwise perfect homology to TRN1 [GenBank accession nos. AAB83973 (896 aa) and AAB71349 (887 aa)]. As a first step, we recloned the coding region of TRN2 from human HeLa cell cDNA and obtained only clones missing the extra sequence. Our TRN2 consensus sequence matches the shorter TRN2 isoform.

To identify additional export substrates of TRN2, we performed pull-down experiments by using HeLa cell extract. 2z-tagged TRN2 and, as a control, 2z-TRN1 were incubated with the cell extract in the absence or presence of RanQ69L(GTP), a GTPase deficient Ran mutant. TRN1/2-associated proteins were then retrieved by using IgG-Sepharose. Surprisingly, a similar set of binding partners was recruited to TRN1/2 (Fig. 1A). The interaction of these proteins with both TRN1/2 was reduced or prevented in the presence of RanGTP, suggesting that these TRN-binding proteins are potential import substrates. Treatment of the HeLa cell extract with ribonuclease did not change the binding pattern, indicating that cellular RNAs do not contribute to the interaction of these proteins with the two different TRN isoforms.

The identities of TRN-bound proteins were determined by MS (Table 1). Among them were already-known TRN1 import substrates such as hnRNP A1 and hnRNP F, additional hnRNP proteins like hnRNP H1 and hnRNP M, and other known RNA-binding proteins. In addition, we identified PQBP-1, which functions as a transcriptional repressor (27). Notably, in all cases analyzed the identical proteins were bound to TRN1/2 (Table 1). For one of these proteins, PQBP-1, a direct interaction with TRN1/2, was confirmed by using recombinant factors (Fig. 8A, which is published as supporting information on the PNAS web site).

HuR and TAP have been described as potential export substrates for TRN2. When we probed the eluates from the pull-down experiment for the presence of HuR, it was detected in both the TRN1/2-bound fractions (Fig. 1B). However, like the TRN1 import substrate hnRNP A1, and unlike export substrates, HuR binding to TRN1/2 was reduced by RanGTP. This suggested that both TRN1/2 might serve as importins for HuR.

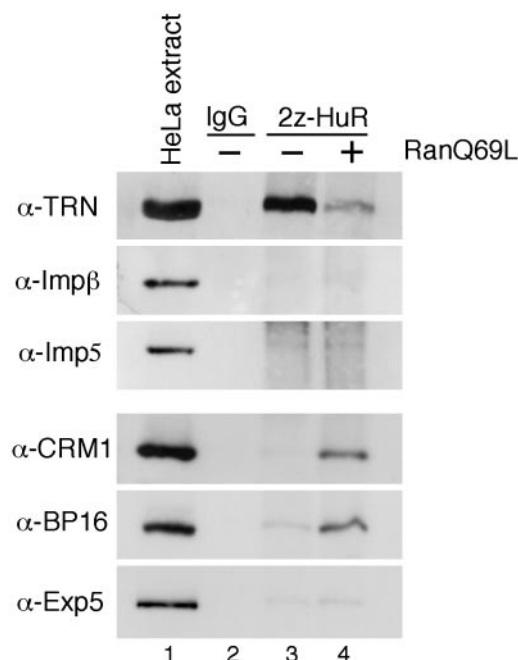


Fig. 2. Identification of transport receptors interacting with HuR. HeLa cell extract was subjected to binding to 2z-HuR in the absence or presence of RanQ69L. Binding to IgG beads (lane 2) served as negative control. Proteins in the bound fractions were analyzed by immunoblotting by using antibodies directed to human TRN1/2, Imp β , RanBP5, RanBP7, CRM1, RanBP16, or Exp5. Load in the input lane equals 1/60 of the load in the bound fractions. Note that under less stringent conditions, Imp β associates with HuR weakly (see Fig. 7).

Similarly, the binding of TAP to TRN2 was RanGTP-sensitive, indicating that TAP may be a TRN2 import substrate (S.G. and U.K., unpublished work).

Next, we characterized the HuR-TRN1/2 interaction. First, we analyzed which nuclear transport receptors in HeLa cell lysate bind to recombinant HuR. Western blot analysis revealed that TRN was enriched on immobilized HuR (Fig. 2). In contrast, other importins like Imp β or -5 were not found among the HuR-binding proteins under these conditions (see also Fig. 7). Again, the interaction between HuR and TRN was disrupted by RanGTP. HuR bound the exportin CRM1 in the presence of RanGTP, confirming that our experiment allows detection of exportin interactions. Interestingly, we also found RanBP16 (23), a RanGTP-binding transport receptor for which transport cargoes have not yet been identified, enriched on HuR in the presence of RanGTP. However, this interaction is likely to have been indirect, because it was sensitive to RNase treatment (data not shown).

The antibody used for detection of TRN was directed against a C-terminal peptide of TRN1. Because this antibody crossreacts with TRN2 (data not shown), we could not discriminate which TRN isoform was recruited to HuR. To distinguish TRN1/2 interaction with HuR, we performed binding reactions by using recombinant factors. TRN1/2 bound to immobilized HuR directly with similar efficiency (Fig. 3A). The HuR nucleocytoplasmic shuttling signal (HNS) domain (15) was necessary and sufficient for this interaction (Figs. 3A and 8A and B). Importantly, binding of both TRN1/2 was sensitive to RanGTP, implying that formation of a complex between TRN2 and HuR in the nucleus should be unlikely. Even preformed complexes between HuR and either of the two TRN isoforms were dissociated by RanGTP (Fig. 3B).

At this point, we could not exclude the possibility that RNA binding of HuR would change the situation and favor the formation of a TRN2-containing export complex. We addressed this point in two different assays.

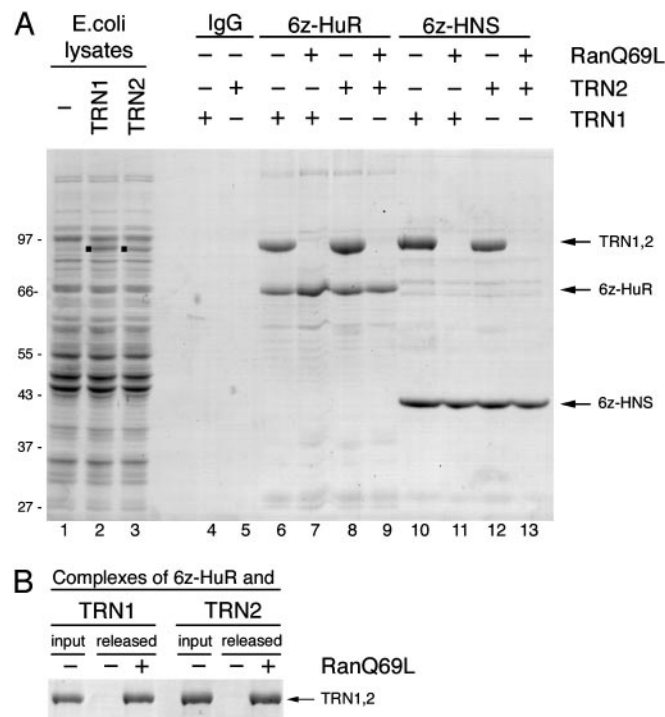


Fig. 3. HuR binds to TRN1/2 directly via its HNS domain in a RanGTP-sensitive manner. (A) *E. coli* lysates were supplemented with equal amounts of purified recombinant TRN1/2 (dots) and subjected to binding to immobilized 6z-HuR or -HNS in the absence or presence of RanQ69L(GTP). Bound proteins were eluted, separated by SDS/PAGE, and detected by Coomassie blue staining. Load in the input lanes equals 1/25 of the load in the bound fractions. (B) Complexes of HuR and TRN1/2 are disassembled by RanGTP. Complexes were performed on IgG-Sepharose (input). Ten minutes after addition of buffer or RanQ69L(GTP), eluted protein was separated from the beads, precipitated, and analyzed by SDS/PAGE. Equal amounts are loaded in all lanes.

First, we used the RanGTPase assay, which allows one to measure apparent dissociation constants (K_d) between RanGTP-binding transport receptors and RanGTP. Importins bind RanGTP strongly, whereas exportins, in the absence of cargo, bind RanGTP weakly. Based on this assay, TRN1 has been shown to bind RanGTP with an apparent $K_d < 1$ nM (28), whereas TRN2 was reported to bind RanGTP weakly (K_d 300 nM) (20). In contrast to these data, we observed that TRN2 bound RanGTP strongly with a subnanomolar K_d (0.6 nM), exactly like TRN1 and Imp β (Fig. 4A).

In the presence of export substrate, the interaction between exportins and RanGTP is stabilized. Importantly, neither the addition of HuR alone nor the presence of an ARE-containing RNA (VEGF-260, part of the 3' untranslated region of VEGF mRNA) that specifically interacts with HuR (ref. 29; Fig. 9, which is published as supporting information on the PNAS web site) increased the affinity of TRN2 for RanGTP (Fig. 4B).

As a second approach, we performed protein gel-shift experiments to determine how RNA binding of HuR would influence the TRN2-HuR interaction. TRN2 formed distinct complexes with RanQ69L(GTP) and HuR that were resolved in nondenaturing gels (Fig. 4C, lanes 2 and 3). Strikingly, the formation of the TRN2/HuR complex was abolished by the addition of VEGF-260 RNA but not by a control RNA (Fig. 4C, lanes 4 and 5). As observed before (Figs. 1B, 2, and 3), the addition of RanGTP prevented the TRN2/HuR complex formation, and the presence of RNA did not prevent this dissociation (lanes 6 and 7). Taken together, these data show that the presence of RNA does not favor the formation of TRN2 containing export

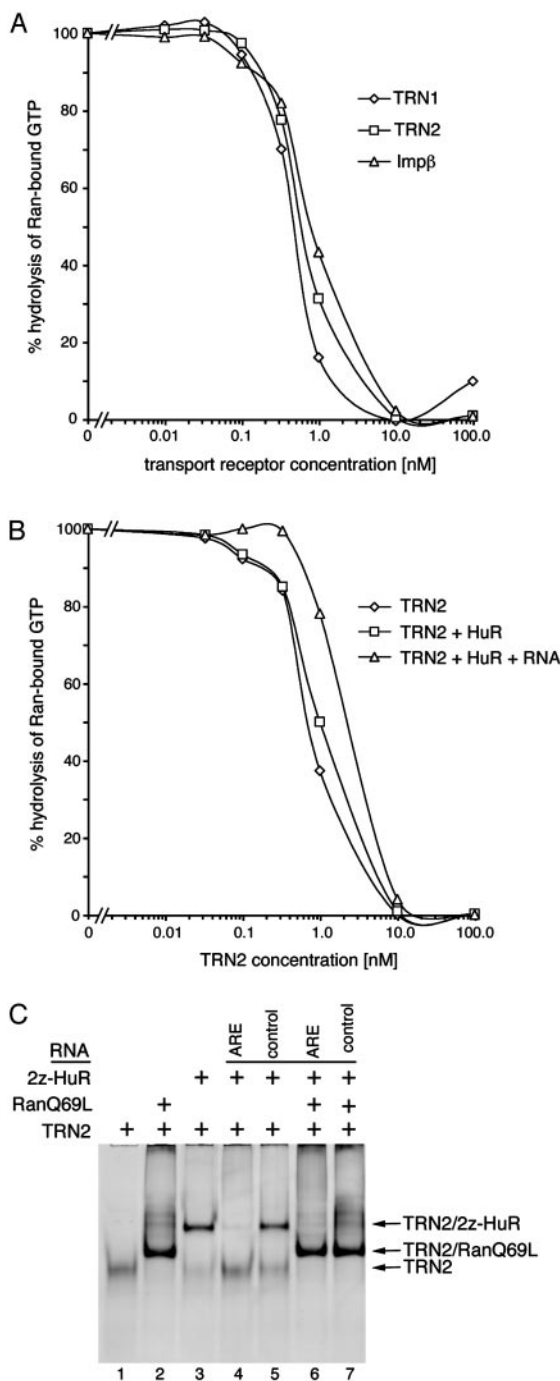


Fig. 4. Characterization of the TRN2/RanGTP interaction. (A) TRN2 binds RanGTP with high affinity. Apparent K_{ds} of complexes between RanGTP and Impβ or TRN1/2 were measured by using the RanGTPase assay. RanGAP-induced GTP hydrolysis by Ran was measured as release of $\gamma^{32}\text{P}_i$ at different concentrations of transport receptors. Each point represents the average of three independent measurements. The apparent K_{ds} are 0.5 nM (TRN1), 0.6 nM (TRN2), and 0.8 nM (Impβ). (B) HuR and RNA do not increase the affinity of TRN2 for RanGTP. HuR (1 μM) and VEGF-260 RNA (2 μM) were added as indicated. (C) Complex formation between TRN2 and HuR or Ran was monitored by native gel electrophoresis and Coomassie blue staining. Preincubation of HuR with an ARE-containing RNA (VEGF-260, lane 4) but not with equal amounts of a control RNA lacking the ARE (VEGF-110, lane 5) prevented the formation of a TRN2/HuR complex. The specific binding of VEGF-260 to 2z-HuR is shown in Fig. 9. Note that another control RNA (part of the actin mRNA coding region, 260 nt in length) behaved as the VEGF-110 control RNA (data not shown). Free HuR and RanQ69L do not migrate into the gel under these conditions, presumably due to their basic isoelectric points.

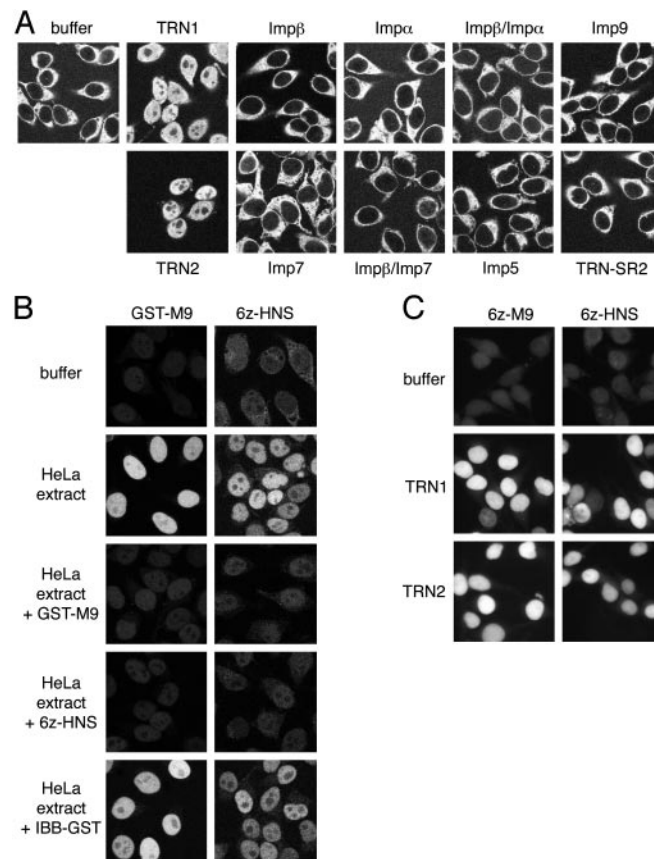


Fig. 5. TRN2 functions as a nuclear import receptor. (A) TRN1/2 mediate nuclear import of HuR. Nuclear import of Alexa-labeled 6z-HuR (2 μM) into the nuclei of semipermeabilized HeLa cells was performed in either buffer alone or in presence of purified transport receptors (1 μM each). After import, samples were fixed and analyzed by confocal microscopy. All transport receptors have been tested for functionality in import (ref. 22 and data not shown). (B) Crosscompetition between M9- and HNS-dependent import pathways. Nuclear import of fluorescent GST-M9 or 6z-HNS (2 μM) was performed in the presence of HeLa extract, Ran, and energy mix. Where indicated, the samples were supplemented with unlabeled competitors (GST-M9, 6z-HNS, or IBB-GST) to a concentration of 10 μM . (C) TRN2 supports nuclear import of 6z-M9 and -HNS. Nuclear import of Alexa-labeled 6z-M9 and -HNS (0.5 μM) was performed in the presence of buffer or TRN1/2 (1 μM each).

complexes. On the contrary, RNA binding to HuR diminishes the TRN2–HuR interaction.

To this point, our data are consistent with a function of TRN2 in nuclear import of HuR. To directly define pathways of HuR import, we examined nuclear uptake of fluorescently labeled HuR in semipermeabilized HeLa cells. Of the tested importins, only TRN1/2 supported nuclear import of HuR (Fig. 5A). Likewise, one of the newly identified TRN2-binding partners, PQBP-1, was imported into the nucleus by both TRN1/2 (Fig. 6B).

In vivo, nuclear import of HuR depends on the HNS domain (15). Also *in vitro*, the HNS domain was necessary and sufficient for nuclear import by TRN1/2 (Fig. 8C). The HNS resembles the M9 nuclear import signal of hnRNP A1 (15, 30–32). To address whether both import signals function in the same manner, we performed competition experiments. Fluorescent fusion proteins containing either the M9 or the HNS domain were imported into the nuclei of semipermeabilized HeLa cells by using a HeLa cell cytoplasmic extract as a source of import factors. Nuclear import of both fusion proteins was similarly competed by the addition of excess unlabeled GST-M9 and 6z-HNS (Fig. 5B). In contrast, the addition of IBB-GST, which

competes for Imp β -mediated import, had no influence. Apparently, M9 and HNS access the same import route. This is confirmed by the observation that TRN1/2 equally stimulate nuclear import of 6z-M9 and 6z-HNS (Fig. 5C).

In summary, our data show that HuR is imported into the nucleus along two nuclear import pathways constituted by the two highly related TRN molecules, TRN1/2.

Discussion

Previous experiments had suggested that TRN1/2 have differing functions, TRN1 as importin for RNA-binding proteins (18) and TRN2 as exportin involved in HuR-associated and general mRNA export (13, 20).

According to our data, also TRN2 serves a primary function in nuclear import of RNA-binding proteins. First, unlike previously reported (20), TRN2 displayed a high affinity for RanGTP comparable to that of other importins like Imp β and TRN1. Second, TRN1/2 interacted with a similar set of RNA-binding proteins in a RanGTP-sensitive manner. Among them are not only hnRNP proteins known to play an important role in mRNA biogenesis such as hnRNP A1, but also the mRNA export factors HuR and TAP. Because complexes between HuR and TRN2 were efficiently dissociated by RanGTP *in vitro*, the same interaction is expected to be disrupted, rather than promoted, in the nuclear compartment. In the case of HuR, RNA binding further diminished its interaction with TRN2, suggesting that it is unlikely that HuR engages in an RNA-containing export complex with TRN2. Third, when tested in *in vitro* nuclear transport assays, TRN1/2 mediated nuclear protein import with comparable efficiencies.

How can the disparity between our findings and previous reports on the function of TRN2 be explained? We have shown that the HuR-HNS competed for nuclear import of hnRNP proteins like hnRNP A1, which are required for mRNP biogenesis. Thus, the previously reported inhibitory effect of HNS peptides on export of ARE-containing RNAs might have been a secondary consequence of a deficiency in nuclear import of hnRNP proteins and potential mRNA export factors including HuR.

In general, nuclear transport signals cause the directional movement of a cargo across the NPC. One can distinguish import from export signals. However, some import signals, referred to as so-called shuttling signals, have been shown to possess export activity when analyzed in interspecies heterokaryon assays. The import activity of these signals is dominant over the export

activity, because reporter proteins fused to these signals are nuclear at steady state. Importantly, the import and export activities of the M9 shuttling signal of hnRNP A1 cannot be separated by mutation; all mutations affect import and export in the same manner (32, 33). Therefore it appears that both M9-dependent import and export are mediated by the same receptor, i.e., TRN. The export activity of an importin probably reflects the reversibility of translocation through the NPC and shows that import signals can, in principle, engage in a reverse import reaction (for discussion, see ref. 2). These shuttling signals (such as the HNS or M9 domains) might be more active in reverse import when isolated from their original protein context. Normally, other protein domains, like RNA-binding domains, contribute to interactions of the transport substrate with other factors (such as RNA) in the cell nucleus and may even promote importin dissociation from these cargoes (34).

Is this reverse export activity of significance for export processes in the cell, such as mRNA export? The M9 domain is not accessible when hnRNP A1 is part of hnRNP particles in the nucleus suggesting that export activity of M9 is unlikely to contribute to mRNA export (19). As we show, the binding of HuR to TRN2 is diminished when HuR is RNA bound, which provides further evidence that TRN2 does not contribute to HuR-associated mRNA export. Interestingly, depletion of TRN2 from cells by RNA interference led to a defect in poly(A)⁺ mRNA export (20). In light of our data, this might also be explained by an indirect effect on a yet-unidentified TRN2 import substrate needed in the nucleus for mRNA export.

Unlike nuclear export of most cellular mRNAs, the export of the ARE-containing *c-fos* mRNA has been suggested to depend on a functional CRM1 export pathway (11, 13). We have observed that CRM1 binds to HuR in the presence of RanGTP from cellular extracts. It is, however, presently unclear how this association is brought about and whether it involves pp32 and APRIL as previously observed (11). The analysis of the molecular mechanism of *c-fos* mRNA export is challenging because we are far from understanding how specific mRNAs like *c-fos* are channeled into a CRM1-dependent export pathway rather than into the conventional TAP-mediated export route.

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