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RNA-Regulated Interaction of Transportin-1 and Exportin-5 with the Double-Stranded RNA-Binding Domain Regulates Nucleocytoplasmic Shuttling of ADAR1▽†

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Double-stranded RNA (dsRNA)-binding proteins interact with substrate RNAs via dsRNA-binding domains (dsRBDs). Several proteins harboring these domains exhibit nucleocytoplasmic shuttling and possibly remain associated with their substrate RNAs bound in the nucleus during nuclear export. In the human RNA-editing enzyme ADAR1-c, the nuclear localization signal overlaps the third dsRBD, while the corresponding import factor is unknown. The protein also lacks a clear nuclear export signal but shuttles between the nucleus and the cytoplasm. Here we identify transportin-1 as the import receptor for ADAR1. Interestingly, dsRNA binding interferes with transportin-1 binding. At the same time, each of the dsRBDs in ADAR1 interacts with the export factor exportin-5. RNA binding stimulates this interaction but is not a prerequisite. Thus, our data demonstrate a role for some dsRBDs as RNA-sensitive nucleocytoplasmic transport signals. dsRBD3 in ADAR1 can mediate nuclear import, while interaction of all dsRBDs might control nuclear export. This finding may have implications for other proteins containing dsRBDs and suggests a selective nuclear export mechanism for substrates interacting with these proteins.

Adenosine deaminases that act on RNA (ADARs) are a family of enzymes that convert adenosines to inosines in structured and double-stranded RNAs (dsRNAs) (3). All ADARs contain a highly conserved catalytic domain at the C terminus and a variable number of dsRNA-binding domains (dsRBDs) upstream of it. In mammals, three members of this protein family have been identified. Of these, only ADAR1 and ADAR2 have been proven to be functionally active, while ADAR3 seems inactive.

ADARs can specifically deaminate single adenosines in a given RNA but can also target multiple adenosines in a promiscuous manner (3, 17). Since inosines are interpreted as guanosines by most cellular processes, the consequences of editing can range from codon alteration to changes in secondary structure and splice sites to site-specific cleavage (17, 37, 40). Editing sites are typically defined by double-stranded structures formed via intramolecular base pairing (29, 32). Recent bioinformatic approaches have shown that editing is a widespread phenomenon altering up to 10% of the human transcriptome, with the majority of editing sites being located in 3′-untranslated regions (2, 4, 19, 22, 28). Also, a number of pri-microRNAs (pri-miRNAs) have been shown to be edited. This can result in both an increase in the repertoire of potential targets and the regulation of miRNA processing (15, 16, 45). Depending on the site of editing, either Drosha or Dicer processing of pri- or pre-miRNAs can be affected. Interestingly, lack of nuclear Drosha processing of pri-miR-142 leads to its degradation by cytoplasmic Tudor-SN, raising the question of how the unprocessed miRNA may get exported from the nucleus (30, 45).

Pri-miRNAs can be edited by either ADAR1 or ADAR2 (30, 45). Of these, at least ADAR1 is a nucleocytoplasmic shuttling protein that could be involved in the transport of substrate RNAs across the nuclear membrane (36, 42). ADAR1 is expressed in two versions: the interferon-induced, 150-kDa ADAR1-i is expressed during viral infection, while the 110-kDa ADAR1-c is constitutively expressed (33, 34). ADAR1-c lacks a bona fide nuclear export signal (NES) but is still able to shuttle between the nucleus and cytoplasm (see Fig. S1 in the supplemental material) (42). We had shown previously that the third dsRBD acts as a nuclear localization signal (NLS), while the first dsRBD promotes cytoplasmic localization, possibly by mediating nuclear export of the protein (42). Mutations that abolish RNA binding of the dsRBDs restore nuclear localization, indicating that RNA binding can modulate the cellular distribution of ADAR1-c (42).

Recently, a few other dsRBDs have been shown to mediate nuclear export. The second dsRBD of interleukin enhancing factor 3 (ILF3) mediates nuclear export in a complex with adenoviral VA1-RNA, RanGTP, and exportin-5 (Exp-5) (5, 12). Similarly, Exp-5 was shown to associate with mammalian Staufen-2 and JAZ in an RNA-dependent manner (7, 24). In these cases, nuclear RNP complex formation, followed by nuclear export and transport within the cytoplasm, has been discussed (18, 24, 25).

The third dsRBD of ADAR1 is the first example of a dsRBD

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with nuclear import activity. This domain is highly homologous to other dsRBDs and shows no significant similarity to any previously identified nuclear import signals. Deletions and chimeric dsRBDs have shown that the NLS domain spans the entire dsRBD (A. Strehblow, unpublished data).

Here we identify transportin-1 (TRN 1) as the nuclear import factor for ADAR1 that specifically recognizes the third dsRBD of this protein. RNA binding by the third dsRBD alone or in combination with other dsRBDs of ADAR1 abolishes TRN 1 binding but promotes Exp-5 binding. We therefore propose an RNA-dependent transport process of ADAR1, where binding of dsRNA inhibits nuclear import of the complex but facilitates its nuclear export.

MATERIALS AND METHODS

Cloning and recombinant protein expression. Glutathione *S*-transferase (GST) fusion constructs containing single or multiple dsRBDs were obtained by digestion of previously published pyruvate kinase fusion constructs (42) and ligation into a pGEX-1 vector containing an extended multiple cloning site which was available in our lab. Exp-5 in pQE60 and TRN 1 in pQE60 were kind gifts of Ian G. Macara and Ulrike Kutay, respectively. His and GST fusion proteins were expressed in *Escherichia coli* BL21(DE3) or *E. coli* XL1-Blue and induced by addition of 1 mM IPTG (isopropyl-β-D-thiogalactopyranoside). Protein purification was then carried out via Ni-nitrilotriacetic acid agarose (Qiagen) and GST-coupled agarose beads (Sigma) following the instructions of the producers. FLAG-His-tagged full-length ADAR1-c was a kind gift of Mary O'Connell.

Preparation of cytosolic and total cell extracts. Cytosolic fractions of HeLa cells were obtained by resuspending the cells in lysis buffer (0.5 mM HEPES, pH 7.3, 0.75 mM magnesium acetate, 0.15 mM EGTA, 3 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride [PMSF]). The suspension was squeezed through a syringe after centrifugation import buffer (20 mM HEPES, pH 7.3, 110 mM potassium acetate, 2 mM magnesium acetate, 0.5 mM EGTA, 1 mM PMSF) was added to the supernatant to a final concentration of 10%.

Heterokaryon fusion assays. Mouse 3T3 cells were transiently transfected with pRAY-myc-*xl*hnRNP A1, using Nanofectin TM (PAA, Linz, Austria), trypsinized, and coplated with HEK293 cells stably transfected with *Homo sapiens* ADAR1-c tagged with green fluorescent protein (hsADAR1-c–GFP). Cells were treated with 40 μg cycloheximide per ml medium for 2 h. Cell fusion was mediated by polyethylene glycol 6000 (1 g/ml serum-free medium) for 2 min, and the cells were then washed and incubated for 6, 8, 18, or 20 h in the presence of cycloheximide. Cells were fixed with 2% paraformaldehyde and 0.05% Triton, permeabilized with methanol, blocked in 10% horse serum, and stained with antibodies diluted in 2.5% horse serum and 1× phosphate-buffered saline. GFP was detected with a rabbit anti-GFP antibody and a secondary goat anti-rabbit Alexa 488-labeled antibody. The myc protein was detected with a monoclonal 9E10 mouse anti-myc antibody and a secondary goat anti-mouse Alexa 568-conjugated antibody. Images were taken with a Zeiss Axioscope microscope using a ×63 objective and a Hamamatsu Orca camera using a Photoshop plug-in.

TRN 1 RNA interference (RNAi). A pool of small interfering RNAs (siRNAs) targeting TRN 1 and the individual oligonucleotides were obtained from Dharmacon RNA Technologies. Transfections were carried out following the manufacturer's instructions. Briefly, HeLa cells were plated to a density of 6×10^5 cells/ml and transfected with siRNAs at a final concentration of 2 μM, using DharmaFECT1. After 72 h, cells were fixed and stained with either a monoclonal mouse anti-TRN 1 antibody (Sigma) or a polyclonal rabbit anti-ADAR1 antibody (Sat309) (available in our lab). Further staining was performed using fluorescently labeled (Alexa 568) goat anti-mouse antibody or fluorescently labeled (Alexa 488) goat anti-rabbit antibody. Detection was executed by confocal fluorescence microscopy (Zeiss) at a magnification of ×63 (numerical aperture, 1.25). Images were recorded with Zeiss LSM software. Cropping and contrast enhancement were performed using Photoshop CSII. For Western blotting, cells were harvested 72 h after transfection by direct lysis in sodium dodecyl sulfate (SDS) sample buffer. Quantification of Western blots was performed with Quantity One software (Bio-Rad).

Import assays. Assays using permeabilized cells were performed following established protocols (1). Briefly, HeLa cells were grown on coverslips and treated with digitonin at a final concentration of 30 μg/ml. Cells were then exposed to 50 nM recombinantly expressed GST-tagged protein and 25 μl cytosolic HeLa extracts (4 mg protein/ml) or 50 nM His-tagged purified karyopherin. rGTP at 1 mM, 1 mM ATP, 20 U/ml creatine phosphokinase, 5 mM creatine phosphate, 0.1% bovine serum albumin, 1 mM PMSF, and import buffer (final concentration, 10%) were added. If necessary, RanQ69L-GTP was added to a final concentration of 150 mM. RanQ69L is a mutant version of Ran that is unable to hydrolyze GTP, even in the presence of cytosolic Ran-GAP. Depletion of the cytosol was done by addition of a monoclonal antibody against TRN 1 (Sigma) to a final concentration of 80 ng/μl. After 20 min at room temperature, the cells were immunostained as mentioned above.

Affinity chromatography. Protein loading, washing, and sealing of a 1-ml HiTrap *N*-hydroxysuccinimide-activated column were performed following the instructions of the producer (Amersham Pharmacia). Briefly, 1.5 mg recombinantly expressed and purified GST-dsRBD3 was loaded onto the column and exposed to a cytosolic extract of HeLa cells (total protein concentration, 0.5 to 1 mg/ml). Elution of potential import factors was achieved by addition of 1 ml RanQ69L-GTP (2 mg/ml). The eluted fractions were separated in a 7.5 to 17% SDS-polyacrylamide gel electrophoresis (SDS-PAGE) gradient gel and visualized via silver staining. Promising fragments were cut out and analyzed via mass spectroscopy.

Immunoprecipitations (IPs). (i) IPs using total cell extracts. To precipitate endogenous TRN 1 from HeLa cells, polyclonal rabbit anti-ADAR1 antibody (Sat 309) (available in our lab) was bound to protein A Sepharose (Amersham Biosciences) and incubated for 1 hour with HeLa total cell extract. Subsequently, the beads were washed thrice with NET2 buffer (150 mM NaCl, 50 mM Tris, pH 7.4, 0.05% NP-40). The beads were resuspended in SDS loading buffer and analyzed via Western blotting. TRN 1 was detected using a monoclonal mouse anti-TRN 1 antibody (Sigma).

(ii) IPs using recombinant proteins. Each protein (75 nM) was bound to GST beads (Sigma) in binding buffer (20 mM HEPES, pH 7.9, 110 mM potassium acetate, 2 mM magnesium acetate, 1 mM EGTA, 1 mM dithiothreitol, 1 mM PMSF, 20 mM NaCl, 5% glycerol, 1% Triton X-100, 14 mM β-mercaptoethanol) in a total volume of 500 μl for 1 h at room temperature. Subsequently, the beads were washed once and resuspended in SDS loading buffer. If required, 400 nM RanQ69L (Jena Bioscience) charged with GTP, 0.1 U RNase V1 (Ambion) per μg protein, or dsRNA at a concentration of 100 μg per reaction was added.

FLIP. U2OS cells were stably transfected with either wild-type ADAR1-c–GFP or a mutant ADAR1-c–GFP carrying mutations in the first and third dsRBDs of ADAR1 (H531A and H754A) that impair RNA binding (20). Clones showing medium expression of the construct were grown on glass coverslip chambers (Labtec) in Dulbecco's modified Eagle's medium. During experiments using fluorescence loss in photobleaching (FLIP), the medium was replaced with HEPES-buffered Dulbecco's modified Eagle's medium and the temperature was maintained at 37°C in a temperature-controlled chamber mounted on a Zeiss LSM 5 Duo microscope. Bleaching was done in 2-min intervals for 30 bleach cycles, with 100% 488-nm laser output (0.4 mW). Before and after each bleach cycle, GFP fluorescence was recorded at 1% 488-nm laser output. To correct for movement of cells on the plate, the bleached region was adjusted every 10 to 20 min. Fluorescence intensity measurements were performed with Zeiss LSM software, using the physiology macro.

RESULTS

ADAR1-c has the ability to shuttle between the nucleus and the cytoplasm. The NLS of ADAR1 overlaps its third dsRBD. However, reporter constructs with all three dsRBDs of ADAR1 are able to accumulate in the nucleus only when RNA binding is inhibited, indicating that RNA binding can modulate nuclear accumulation of ADAR1-c (42). To clarify whether full-length ADAR1-c would also respond in a similar manner and to distinguish whether RNA binding prevents nuclear import or promotes nuclear export, we tested if ADAR1-c was able to shuttle between the nucleus and cytoplasm. Indeed, in heterokaryon assays, stably transfected GFP-tagged hsADAR1-c was able to shuttle from the human to the mouse nucleus. Transiently transfected clones expressing myc-tagged *Xenopus laevis* hnRNP A1 protein and hnRNP-C were used as positive and negative controls, respectively. While *X. laevis* hnRNP A1 showed equal distributions in nuclei of both cell types 4 h after cell fusion, ADAR1-c required as much as 8 hours to become fully equili-

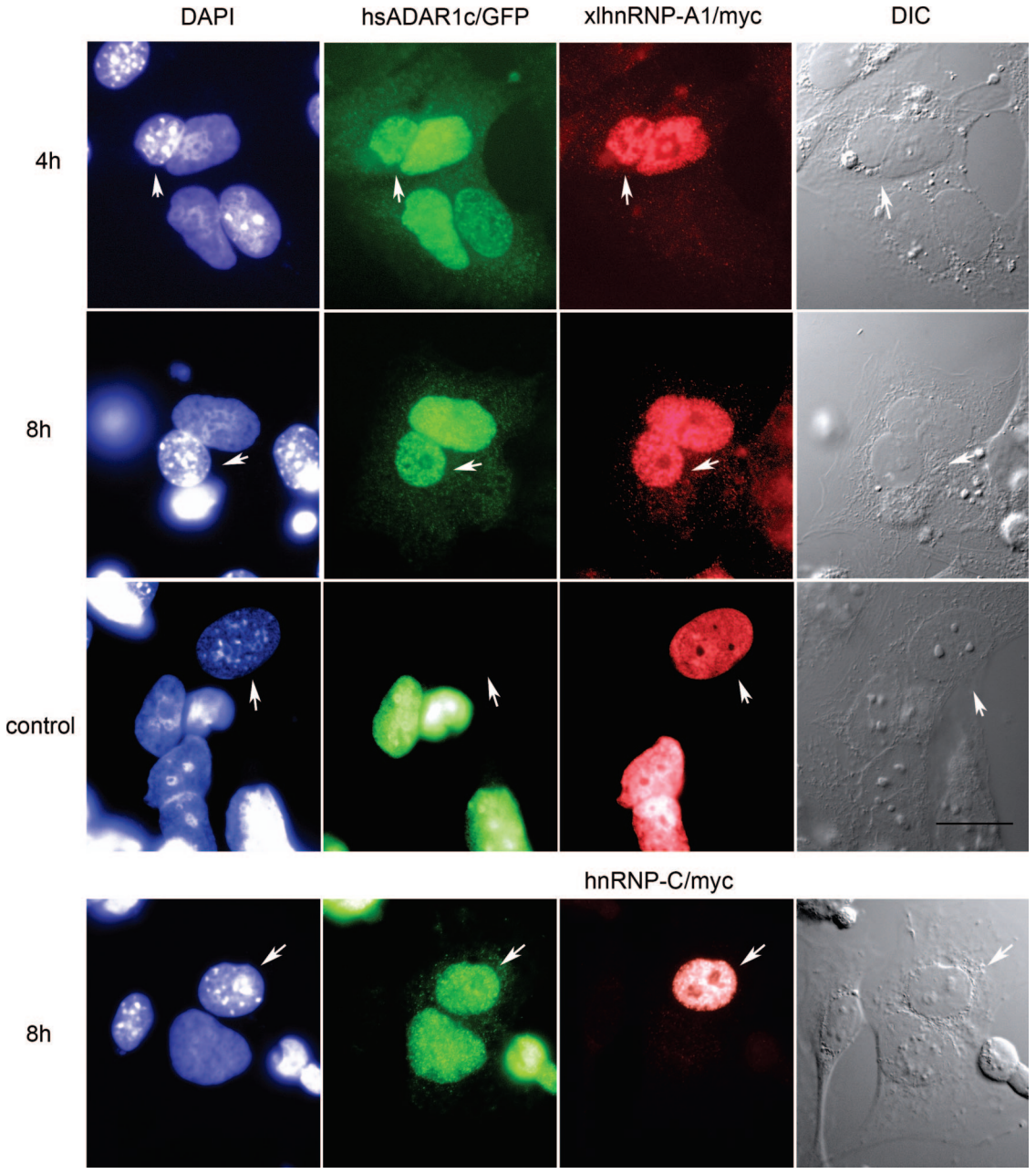
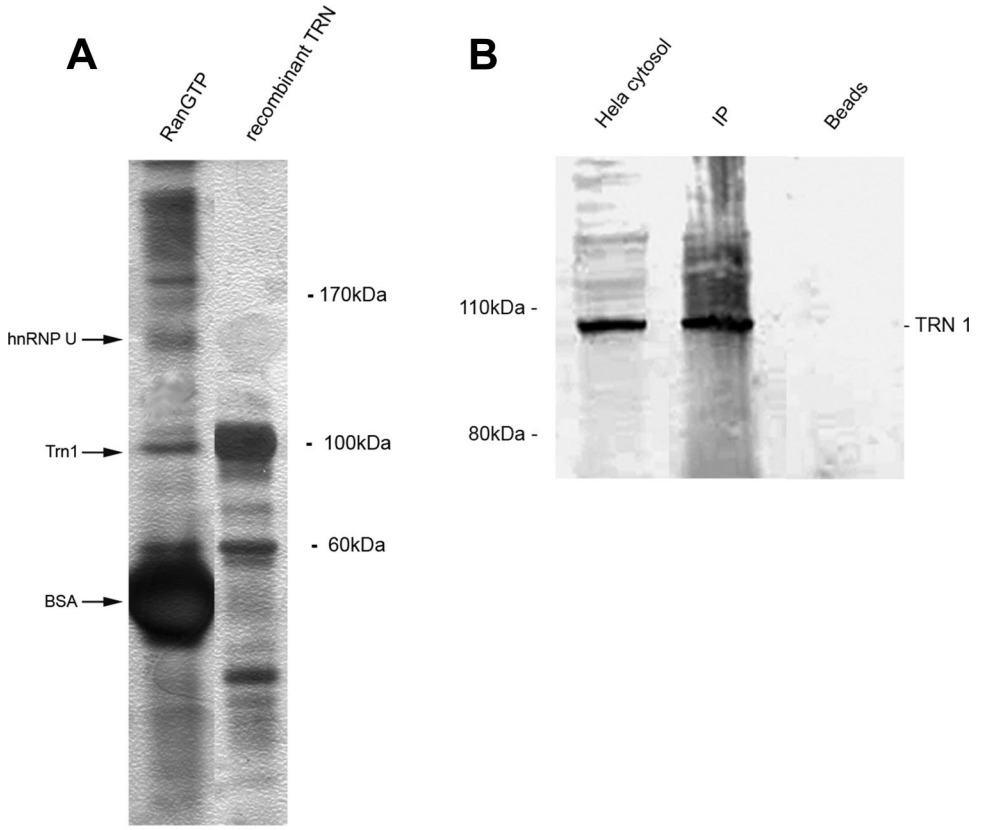


FIG. 1. The p110 version of ADAR1 is a shuttling protein. To test the shuttling ability of the short, constitutively expressed version of ADAR1, ADAR1-c was GFP tagged at the C terminus and stably transfected into HEK293 cells. Stably transfected cells were fused with mouse 3T3 cells that were transiently transfected with myc-tagged hnRNP A1 (top three rows) or the nonshuttling myc-tagged hnRNP C (bottom row). Cell fusion and subsequent incubation were performed in the presence of cycloheximide. Cells were fixed and stained after 4 and 8 h for the presence of either protein. Mouse nuclei were identified by brilliant DAPI (4′,6-diamidino-2-phenylindole)-positive heterochromatin foci and are indicated by arrows. While the hnRNP A1 protein readily moved from the mouse nucleus to the human nucleus within 4 h, analysis of several experiments indicated that ADAR1-c moved with slightly slower kinetics but was well detectable in the mouse nucleus after 4 and 8 h. Within the same time frame, the nonshuttling protein hnRNP C remained in the mouse nucleus (bottom row). In unfused cells, no movement of either protein could be detected (control). Bar = 20 μm. DIC, differential interference contrast.

brated between the two nuclei (Fig. 1). These results demonstrate that the short version of human ADAR1 is able to shuttle between the nucleus and the cytoplasm despite its lack of an obvious NES, with relatively slow kinetics.

TRN 1 binds human ADAR1 via its third dsRBD. The third dsRBD of hsADAR1 was the first dsRBD shown to act as a nuclear import signal (10). Chimeric constructs generated between this and other dsRBDs all failed to accumulate in the nucleus, precluding identification of a short region within this dsRBD essential for nuclear import. Also, several known import receptors, such as importin α, importin β, and TRN SR, all failed to interact with this dsRBD NLS



C

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  1 MEYEWKPDEQ GLQQILQLLK ESQSPDTTIQ RTVQQKLEQL NQYPDFNNYL
 51 IFVLTKLKSE DEPTRSLSGL ILKNNVKAHF QNFPNGVTDF IKSECLNNIG
101 DSSPLIRATV GILITTIASK GELQNWPDLL PKLCSLLDSE DYNTCEGAFG
151 ALQKICEDSA EILDSDVLDR PLNIMIPKFL QFFKHSSPKI RSHAVACVNQ
201 FIISRTQALM LHIDSFTENL FALAGDEEPE VRKNVCRALV MLLEVRMDRL
251 LPHMHNIVEY MLQRTQDQDE NVALEACEFW LTLAEQPICK DVLVRHLPKL
301 IPVLVNGMKY SDIDIILLKG DVEEDETIPD SEQDIRPRFH RSRTVAQQHD
351 EDGIEEEDDD DDEIDDDDTI SDWNLRKCSA AALDVLANVY RDELLPHILP
401 LLKELLFHHE WVVKESGILV LGAIAEGCMQ GMIPYLPELI PHLIQCLSDK
451 KALVRSITCW TLSRYAHWVV SQPPDTYLKP LMTELLKRIL DSNKRVQEAA
501 CSAFATLEEE ACTELVPYLA YILDTLVFAF SKYQHKNLLI LYDAIGTLAD
551 SVGHHLNKPE YIQMLMPPLI QKWNMLKDED KDLFPLLECL SSVATALQSG
601 FLPYCEPVYQ RCVNLVQKTL AQAMLNNAQP DQYEAPDKDF MIVALDLLSG
651 LAEGLGGNIE QLVARSNILT LMYQCMQDKM PEVRQSSFAL LGDLTKACFQ
701 HVKPCIADFM PILGTNLNPE FISVCNNATW AIGEISIQMG IEMQPYIPMV
751 LHQLVEIINR PNTPKTLLEN TAITIGRLGY VCPQEVAPML QQFIRPWCTS
801 LRNIRDNEEK DSAFRGICTM ISVNPSGVIQ DFIFFCDAVA SWINPKDDLR
851 DMFCKILHGF KNQVGDENWR RFSDQFPLPL KERLAAFYGV
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FIG. 2. TRN 1 interacts with ADAR1 in vitro and in vivo via the third dsRBD. (A) To identify potential import factors binding the third dsRBD of ADAR1, affinity chromatography of HeLa cell cytosol was performed on immobilized recombinantly expressed dsRBD3. Potential import receptors were eluted by RanQ69L-GTP, the fragments indicated by arrows were cut out of a silver-stained gel, and the band of 100 kDa was identified as TRN 1 by mass spectrometry. As a reference, 0.5 μg of recombinantly expressed TRN 1 was loaded. (B) To prove the in vivo interaction of ADAR1 with TRN 1, HeLa cell cytosol was used for immunoprecipitation using hsADAR1-specific antibodies. The precipitated material was probed for the presence of TRN 1 by Western blotting. Total HeLa cell cytosol was loaded as a reference. TRN 1 could not be detected when beads without an ADAR1-specific antibody were used. (C) Peptides identified by mass spectrometry are highlighted in the TRN 1 sequence (underlined and italic).

(data not shown). To identify the import receptor binding to dsRBD3, the domain was recombinantly expressed and used for affinity chromatography. HeLa cell cytosol was allowed to bind to the column, and import factors were eluted with the nonhydrolyzing mutant RanQ69L-GTP (26). Separation of the eluted proteins and subsequent mass spectrometry identified a protein of 90 kDa as TRN 1 (karyopherin β2A) (Fig. 2A and C). To demonstrate the ability of TRN 1 to interact with full-length ADAR1 in vivo, hsADAR1 was immunoprecipitated from HeLa cell lysates and tested for association with TRN 1 by Western blotting of immunoprecipitated material. Indeed, TRN 1 could be coprecipitated with ADAR1, indicating an interaction of the two proteins in vivo (Fig. 2B).

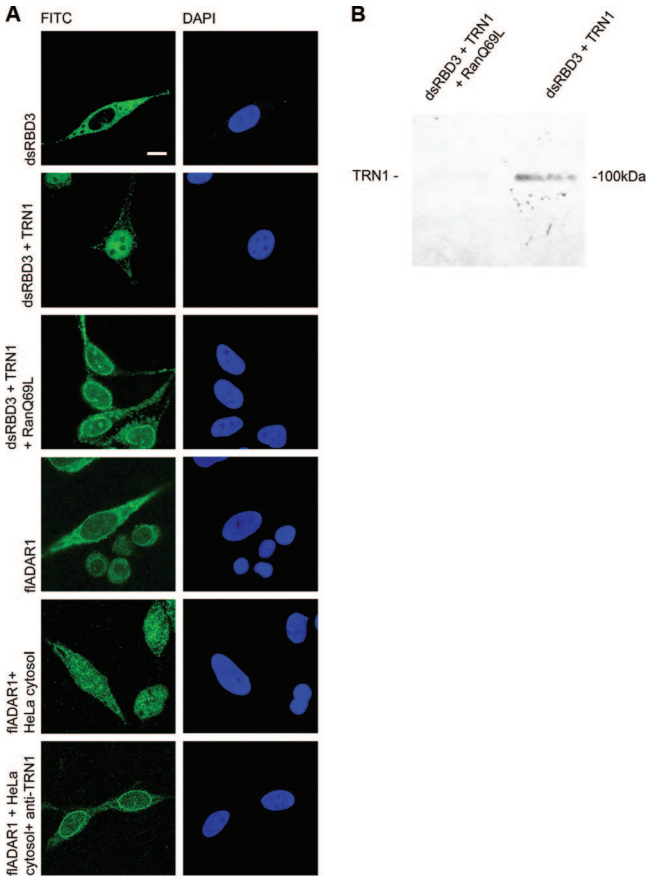

FIG. 3. TRN 1 imports dsRBD3 and hsADAR1 into the nucleus in a RanGTP-dependent manner. (A) In permeabilized cell import assays, TRN 1 was able to mediate nuclear accumulation of recombinantly expressed dsRBD3 of hsADAR1 without additional cofactors (dsRBD3 + TRN1). The addition of nonhydrolyzable RanGTP (RanQ69L) led to a mainly cytoplasmic localization of the protein (dsRBD3 + TRN 1 + RanQ69L). Full-length ADAR1c was partially imported into the nucleus by HeLa cell cytosol (flADAR1 + HeLa cytosol). Incubation of HeLa cell cytosol with a monoclonal antibody directed against TRN 1 abolished nuclear import of ADAR1 (flADAR1 + HeLa cytosol + anti-TRN 1). Bar = 20 μm. (B) Interaction between TRN 1 and dsRBD3 was also shown in a biochemical pull-down assay in which dsRBD3 was coupled to beads and exposed to TRN 1 (dsRBD3 + TRN 1). The addition of RanGTP abolished the interaction between the proteins (dsRBD3 + TRN 1 + RanGTPQ69L). Precipitated His-tagged TRN 1 was detected by means of an anti-His antibody.

TRN 1 mediates nuclear import of hsADAR1-dsRBD3 in a RanGTP-dependent manner. To test whether TRN 1 is able to import dsRBD3 into the nucleus, import assays were performed with digitonin-permeabilized HeLa cells depleted of their cytoplasmic transport machinery (1). Permeabilized cells were incubated with recombinantly expressed, epitope-tagged dsRBD3, His-tagged TRN 1, and an energy source. Subsequent staining of dsRBD3 showed that the addition of TRN 1 leads to complete nuclear accumulation of dsRBD3, indicating that TRN 1 is able to mediate nuclear import without the need for additional cofactors (Fig. 3). Addition of RanQ69L-GTP prevented import of dsRBD3, indicating that TRN 1 and dsRBD3 indeed display all characteristics of a functional import complex (Fig. 3). Binding of dsRBD3 to TRN 1 was also shown biochemically in pull-down assays. The dsRBD fusion was coupled to glutathione Sepharose and incubated with His-tagged TRN 1 in the presence or absence of RanGTP. Subsequent staining of the precipitated material with an anti-His antibody proved a RanGTP-sensitive interaction of dsRBD3 and TRN 1 (Fig. 3).

dsRBDs are highly conserved. Therefore, to determine the specificity of the interaction between TRN 1 and dsRBD3, import and pull-down assays using the first or second dsRBD of hsADAR1 were performed. dsRBD1 and dsRBD2 show high levels of homology to dsRBD3 yet possess no NLS activity (42). Consistently, both dsRBD1 and dsRBD2 of ADAR1 failed to be transferred to the nucleus by TRN 1 (see Fig. S2 in the supplemental material). Similarly, no interaction between dsRBD1 or dsRBD2 of ADAR1 and TRN 1 could be observed in pull-down assays (see Fig. S2 in the supplemental material).

TRN 1 is required to mediate nuclear transport of ADAR1. Having shown that TRN 1 is able to specifically interact with and mediate nuclear transport of a construct harboring dsRBD3, the active NLS in human ADAR1, we next set out to determine whether TRN 1 was essential for nuclear transport of human ADAR1. Two assays were used to demonstrate the essential role of TRN 1 in nuclear transport of ADAR1. First, recombinant hsADAR1c-FLAG or dsRBD3-GST was used in permeabilized cell import assays using total HeLa cell cytosol as the source of import factors. As depicted in Fig. 3, HeLa cell cytosol was able to mediate nuclear import of ADAR1-c or dsRBD3-GST (not shown). The addition of a TRN 1-specific monoclonal antibody prevented nuclear transport, leading to a cytoplasmic and perinuclear localization of ADAR1-c or dsRBD3-GST, illustrating the crucial role of TRN 1 in nuclear import (Fig. 3 and data not shown).

As a second line of evidence, to demonstrate that TRN 1 is the sole import factor for hsADAR1, TRN 1 was knocked down in HeLa cells, using individual siRNAs or an siRNA pool against TRN 1 (Fig. 4). Three of the four individual siRNAs and the SMART pool led not only to a severe decrease in the concentration of TRN 1 but also to a cytoplasmic localization of ADAR1 72 h after RNAi treatment (Fig. 4). Transfection with a pool of control siRNAs showed no alteration in TRN 1 levels or ADAR1 localization (Fig. 4). Taken together, the two sets of experiments provide strong evidence that TRN 1 is the sole import factor for human ADAR1.

Influence of dsRNA binding on cellular organization of hsADAR1. To investigate the RNA-binding-dependent inhibitory effect of the first dsRBD on the NLS activity of the third dsRBD, import assays using a tagged fusion construct of dsRBD1 and dsRBD3 were performed. Consistent with previous transfection experiments, TRN 1 was not able to mediate nuclear import of this construct (dsRBD1+3) (Fig. 5A). However, simultaneous introduction of mutations that abolished RNA binding in both dsRBDs (A531H, H745A, E755C, and F758A) restored its nuclear import by TRN 1 (Fig. 5A).

As discussed previously, this finding is consistent with either cytoplasmic anchoring of dsRBDs by an RNA or, alternatively, RNA-dependent inhibition of TRN 1 binding. We therefore tested the influence of RNA binding on the interaction between different dsRBD constructs and TRN 1 in pull-down assays using equimolar amounts of all components.

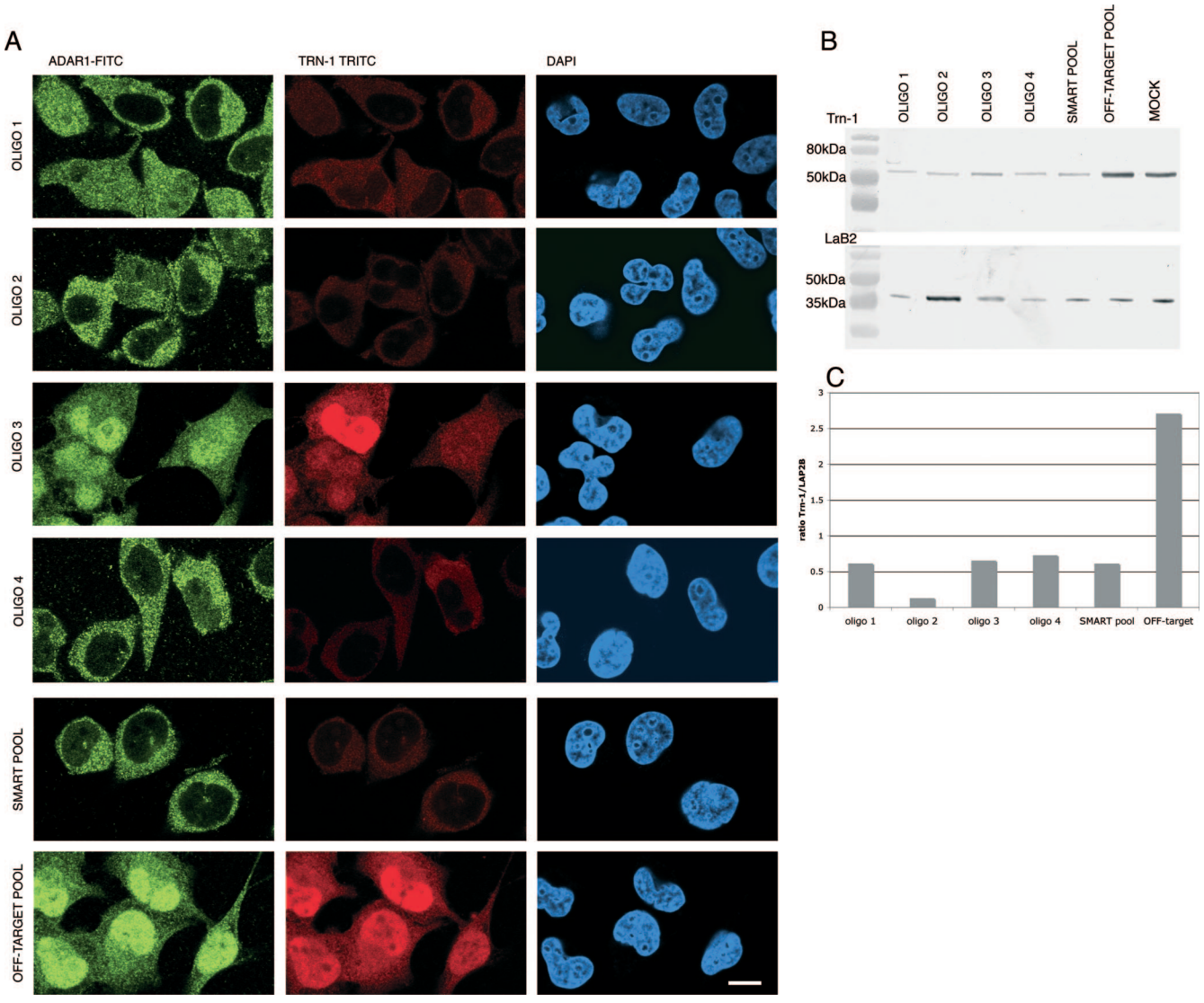

FIG. 4. TRN 1 is required for nuclear import of human ADAR1 in vivo. To determine the role of TRN 1 in nuclear transport of ADAR1, in vivo knockdown experiments with individual siRNAs and an siRNA pool directed against TRN 1 were performed. (A) Immunofluorescence of HeLa cells transfected with siRNAs directed against TRN 1 shows a drastic decrease in the concentration of TRN 1 and a loss of nuclear localization of ADAR1 (Oligo 1 to Oligo 4 and SMART pool). Transfection with scrambled oligonucleotides that showed no homology to endogenous targets had no effect on the concentration of TRN 1 or the cellular localization of ADAR1 (off-target pool). Bar = 10 μm. (B) Reduction of TRN 1 expression upon siRNA treatment was monitored by Western blotting. As a loading control, the Western blot was also probed with an antibody against lamin B2 (LaB2). (C) Quantification of the ratios of Western blot signals for TRN 1 to those for lamin B2 shows a clear reduction of TRN 1 in all siRNA-treated samples.

The addition of dsRNA completely inhibited binding of TRN 1 to dsRBD3, whereas both proteins were able to interact in the absence of RNA (Fig. 5B). A single amino acid exchange in dsRBD3 that reduced RNA binding, combined with RNase V1 treatment to remove residual dsRNA, led to an even stronger interaction with TRN 1 than that observed for untreated wild-type dsRBD3, indicating that dsRNA binding and interaction of dsRBD3 with TRN 1 are mutually exclusive (Fig. 5B). The fusion construct of dsRBD1 and dsRBD3 bound TRN 1 very weakly, and the interaction was completely abolished upon addition of dsRNA. A mutation in the first dsRBD that reduced RNA binding showed a significantly stronger interaction with TRN 1 than that with the wild-type construct. However, the interaction was still sensitive to the addition of dsRNA. The construct carrying mutations that inhibited RNA binding in dsRBDs 1 and 3 also displayed a strong interaction with TRN 1 that was insensitive to the addition of dsRNA (Fig. 5).

A direct fusion of dsRBD1 to dsRBD3 is artificial and does not occur in wild-type ADAR1. Therefore, a construct containing all three dsRBDs (ds1-3) was tested as well. As expected, this construct showed a strong interaction with TRN 1. The addition of dsRNA led to a 75% reduction of this interaction (Fig. 5), indicating that RNA binding can also disrupt the interaction of dsRBD3 and TRN 1 when the RNA-binding domains occur in their natural context. The specificity of all

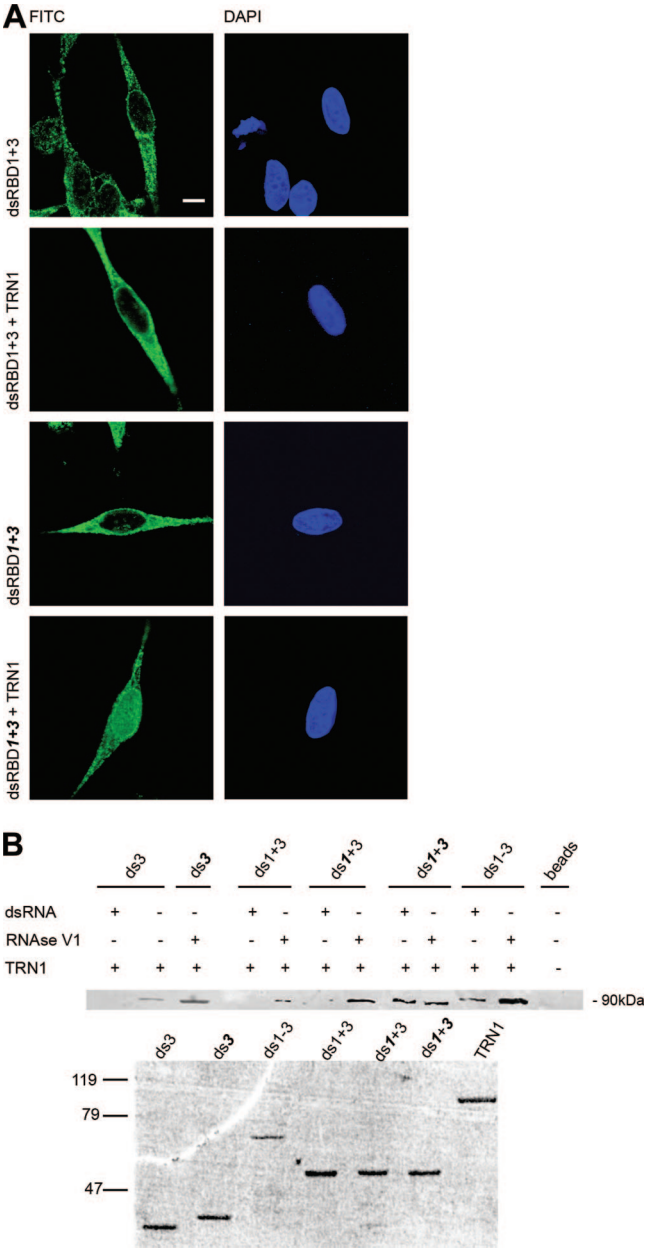

FIG. 5. The first dsRBD of ADAR1 inhibits binding of TRN 1 in an RNA-dependent manner. (A) TRN 1 fails to mediate nuclear accumulation of a protein consisting of dsRBD1 and dsRBD3 of human ADAR1 (dsRBD1+3 + TRN 1). When the RNA-binding ability of both dsRBDs was reduced by mutation, TRN 1 could import the resulting protein into the nucleus (dsRBD***1+3*** + TRN 1). Bar = 20 μm. (B) TRN 1 can bind constructs containing one or several ADAR1 dsRBDs in vitro. The addition of dsRNA strongly reduced or even inhibited TRN 1 binding. On the other hand, constructs that showed no or reduced RNA-binding ability due to mutations in their dsRBDs (indicated by bold italic domain numbers, e.g., dsRBD***3***, dsRBD***1***+3, and dsRBD***1+3***) were less sensitive or even insensitive to the addition of dsRNA. Additional digestion of dsRBDs with RNase V1 enhanced their interaction with TRN 1. The proteins coupled to the beads in the pull-down assay were quantified and checked for integrity on a Coomassie-stained SDS-PAGE gel.

interactions observed was tested by their sensitivity to the addition of RanGTP (data not shown). Taken together, these results strongly argue for a mutually exclusive interaction of dsRBD3 with either TRN 1 or dsRNA.

Exp-5 binds the dsRBDs and interferes with TRN 1 activity. ADAR1-c lacks a well-defined NES yet is able to shuttle between the nucleus and cytoplasm. Thus, the questions of which domain could serve as an NES and which export receptor might mediate nuclear export remained. As discussed above, the presence of dsRBD1 and, to a somewhat lesser extent, dsRBD2 interferes with nuclear accumulation of the NLS-bearing dsRBD3 in an RNA binding-dependent manner in vivo. This suggests that some dsRBDs or the entire RNA-binding region might mediate nuclear export (42). Consistent with this idea, Exp-5 was reported to mediate nuclear export of dsRBD-containing proteins, possibly via a dsRNA bridge (5, 7, 12, 24). We therefore tested whether Exp-5 could also interact with the dsRBDs of ADAR1. As a positive control, we included dsRBD2 of ILF3, a known interaction partner of Exp-5, in our experiments. dsRBD2 of ILF3 showed an RNA-independent but RanGTP-dependent interaction with Exp-5 (Fig. 6A). Similarly, dsRBD1, dsRBD2, or dsRBD3 of ADAR1 did interact with Exp-5, in a RanGTP-dependent manner. The addition of dsRNA stimulated the interaction of dsRBD1 with Exp-5 but was not essential, as digestion with RNase V1 did not abolish this interaction (Fig. 6B). Interestingly, a construct containing all three dsRBDs in their natural organization (dsRBD1-2-3) showed a weak interaction with Exp-5 even in the absence of RanGTP. This interaction is RNA dependent, since it was strengthened by the addition of dsRNA but strongly reduced upon digestion with RNase V1. However, the addition of RanGTP most profoundly stimulated the interaction between dsRBD1-2-3 and Exp-5. In the presence of RanGTP, the influence of dsRNA became almost negligible (Fig. 6C). This indicates that within the cell nucleus, where a high RanGTP concentration can be found, dsRBDs can interact with Exp-5. dsRNA has a stimulatory effect on this interaction but is not a prerequisite. Obviously, the more dsRBDs are present in a construct, the stronger the influence of bound RNA becomes.

Both Exp-5 and TRN 1 interact with the RNA-binding region of ADAR1 and react upon the addition of dsRNA. It thus appeared possible that binding of one factor would compete with binding of the other in an RNA-regulated manner. Therefore, to test whether Exp-5 would interfere with TRN 1, import assays were performed with both TRN 1 and Exp-5. As shown in Fig. 6E, import of dsRBD3 was inhibited when equimolar amounts of TRN 1 and Exp-5 were added to the reaction mix. Conceptually, the cytoplasmic localization of dsRBD3 could be interpreted as a prevention of import or, alternatively, as TRN 1-mediated import followed by rapid export via Exp-5. However, since no RanGTP was added in these experiments and moderate binding of Exp-5 to dsRBDs was observed even in the absence of RanGTP, inhibition of import by competitive binding of Exp-5 to the NLS/NES region appears more likely.

Finally, we wanted to determine the influence of RNA binding on the shuttling behavior of ADAR1-c. Could it be that RNA binding stimulates export while inhibiting import? To address this question, FLIP experiments were performed with cell lines stably expressing either wild-type ADAR1-c or mutated ADAR1-c–GFP in which both the first and third RBDs were mutated to yield a protein in which RNA binding was reduced at least 70% (10a). U2OS cells were chosen for their flat appearance and large cytoplasmic extensions to generate

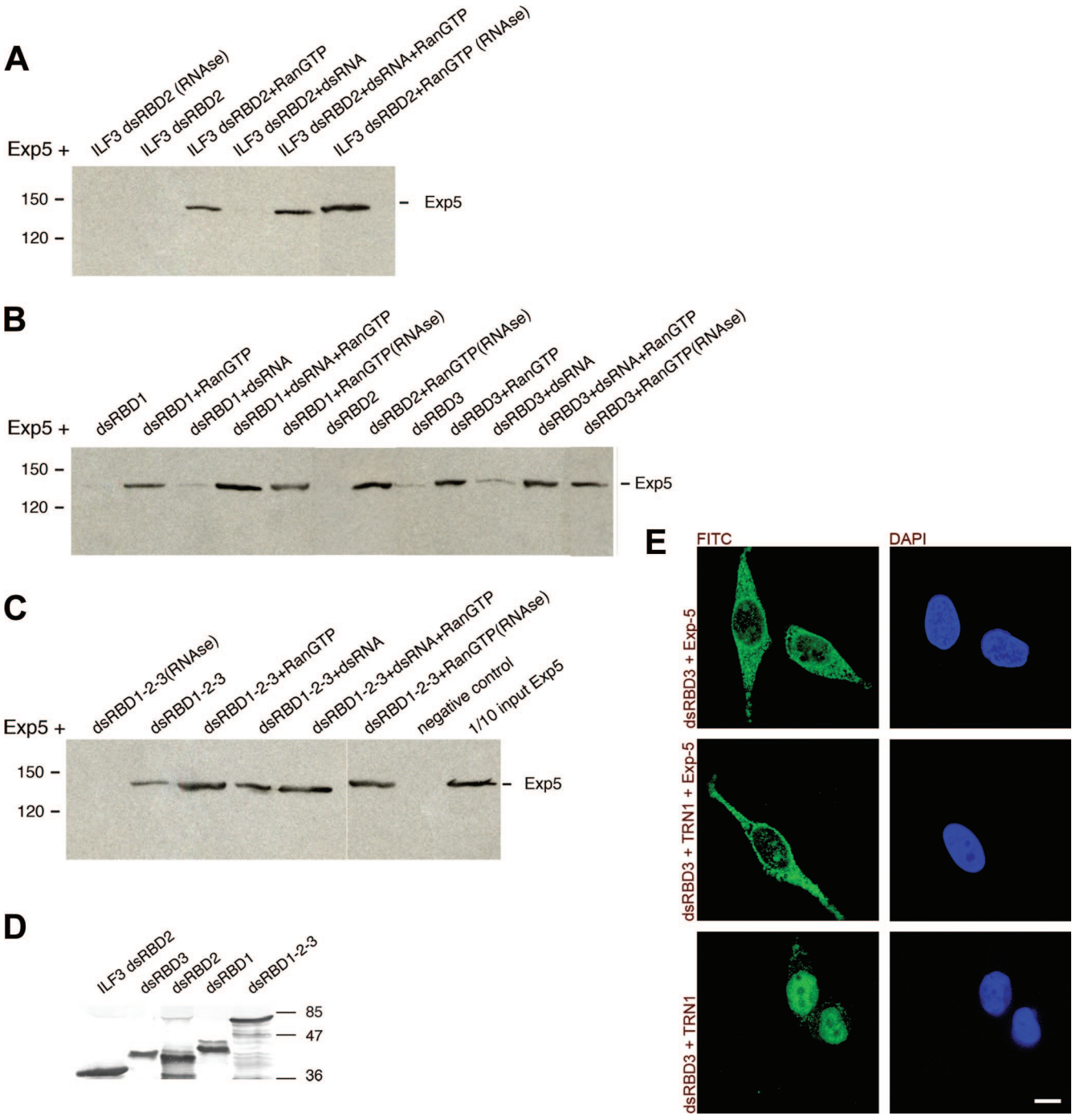

FIG. 6. Exp-5 binds the dsRBDs of ADAR1 in a RanGTP-dependent manner and inhibits TRN 1-mediated nuclear import of dsRBD3. (A) His-tagged, recombinantly expressed Exp-5 was used in pull-down assays, using the GST-tagged second dsRBD of ILF3 as a positive control. Exp-5 that bound to the dsRBDs was detected by Western blotting. The interaction with ILF3 was RanGTP dependent and was slightly negatively influenced by the presence of dsRNA. (B) Exp-5 can interact with all individual dsRBDs of ADAR1 in a RanGTP-dependent manner. The addition of dsRNA had a slight stimulatory effect on the interaction of Exp-5 with the first dsRBD of ADAR1. Interaction with the other individual dsRBDs seemed unaffected by the addition of dsRNA. (C) Exp-5 can bind a construct containing all three dsRBDs of ADAR1 in a RanGTP- and dsRNA-dependent manner. dsRNA was sufficient to allow an interaction between the RNA-binding region of ADAR1 and Exp-5. Treatment with RNase V1 inhibited this interaction. RanGTP had the strongest stimulatory effect on this interaction. No further stimulation of the interaction was observed when dsRNA was added on top of RanGTP. (D) Recombinant proteins used in the pull-down assays were checked for integrity and quantified on a Coomassie-stained SDS-PAGE gel. (E) Exp-5 can interfere with TRN 1 activity in permeabilized cell import assays. Equimolar addition of Exp-5 to a TRN 1-containing import mix efficiently inhibited nuclear import (dsRBD3 + TRN 1 + Exp-5), while the TRN 1 addition alone led to nuclear import of the cargo. Exp-5 itself showed no activity in import assays.

the stable clones used in FLIP experiments. At least two moderately expressing cell lines were chosen for each construct. About 30% of a cell's cytoplasm was bleached with maximum laser intensity at 2-min intervals, followed by recording of GFP fluorescence of the corresponding nucleus and a reference nucleus in the same field. Consistent with the nucleocytoplasmic shuttling of ADAR1-c, a loss of nuclear fluorescence was observed upon cytoplasmic bleaching (see Fig. S3A in the supplemental material). The rate of fluorescence loss and thus the speed of shuttling were only moderately faster for wild-type ADAR1-c than for the mutated version showing reduced RNA-binding ability. This indicates that while RNA binding stimulates export, it is not a prerequisite for shuttling, at least under conditions where the amount of substrate RNAs bound by ADAR1-c might be limited.

DISCUSSION

TRN 1 mediates nuclear import of hsADAR1 in the absence of dsRNA. dsRBDs can be found in a wide variety of proteins and are thus involved in the localization, processing, stability, and editing of RNA (6). Moreover, dsRBDs can also accomplish functions unrelated to RNA binding (9, 39) and can, for instance, influence the cellular distribution of the protein they reside in. Mammalian Staufen 2, the protein JAZ, and ILF3 are all exported from the nucleus via the interaction of one of their dsRBDs with Exp-5 (7, 12, 24).

The third dsRBD of hsADAR1 was the first dsRBD to be identified as an NLS that is both necessary and sufficient for nuclear import of ADAR1 (10). Mutational analysis of the third dsRBD of ADAR1 has shown that amino acids required for NLS activity are spread throughout the entire dsRBD. This in turn suggests that a characteristic structure, rather than a certain stretch of primary amino acids, is recognized by an import receptor.

We tested several import factors, including importin α, TRN SR, and importin 9, for the ability to interact with the NLS in ADAR1, but all failed to do so. Only affinity purification allowed us to identify TRN 1 as a RanGTP-sensitive import receptor of the third dsRBD in ADAR1. TRN 1 is distantly related to importin β and was shown to act as a nuclear import factor for the M9-containing protein hnRNP A1, hnRNP D, TAP, and ribosomal protein L23a (14, 35, 41, 43, 44). The interaction between the hnRNP A1 M9 domain and TRN 1 involves a structurally disordered NLS with an overall positive charge and an R/K/HX$_{2-5}$PY consensus sequence (21). Calculations of the electrostatic surface potentials of both proteins mapped the substrate binding site for the M9 domain to the C terminus of TRN 1. The interaction is believed to occur between the positively charged NLS and the negatively charged interior of the TRN 1 C-terminal arch (8). Structural analysis has recently shown that two surfaces consisting of HEAT repeats are involved in the tight and specific recognition of substrates. While one surface makes the initial contact with the substrate, the second surface is involved in tight binding of the substrate and in RanGTP-dependent substrate release (13). While basic in nature, the third dsRBD of ADAR1 differs from other substrates recognized by TRN 1. It possesses a complex secondary structure and lacks the predicted consensus sequence. Further experiments will therefore be required to identify the mechanism of interaction between this dsRBD and TRN 1.

Along these lines, it will be interesting to clarify how TRN 1 selectively recognizes the third dsRBD of ADAR1 while no interaction with other dsRBDs can be detected.

dsRBD3 not only mediates nuclear import of ADAR1 but also is required for RNA binding. Binding of dsRNA effectively blocks the interaction of dsRBD3 with TRN 1. Recognition of the sugar-phosphate backbone of dsRNA by a dsRBD involves its β1-β2 loop as well as the amino-terminal part of α-helix 2. Most likely, RNA binding also leads to a structural change of the dsRBD (38). Therefore, RNA binding could lead to a change in the exposure of amino acids needed to mediate interaction with TRN 1, leaving them inaccessible for the import factor. Alternatively, amino acids normally used to interact with dsRNA could provide the basic surface that is required for binding of TRN 1. In this case, RNA binding would block the interaction sites for the import receptor.

However, dsRBD3 alone binds RNA only weakly, allowing the observed interaction with TRN 1 in vitro and in import assays. The presence of additional dsRBDs inhibits binding of TRN 1, most likely by increasing the interaction of the third dsRBD with dsRNA. We consistently showed that mutations that reduce RNA binding, but also RNase V1 treatment, restore the interaction of dsRBD3 with TRN 1, even if a second dsRBD is present in the construct. In vivo, interaction between ADAR1 and TRN 1 occurs in the cytoplasm. The sensitivity of this interaction to dsRNA implies that ADAR1 can be imported into the nucleus only when no RNA is bound. This mechanism might prevent nuclear import of RNAs that associate with ADAR1 in the cytoplasm.

Exp-5 and TRN 1 interfere with each other for dsRBD binding. Heterokaryon assays have shown that ADAR1-c p110 can be exported from the nucleus despite the absence of a defined NES. Our experiments presented here suggest that Exp-5 plays a pivotal role in this nuclear export. Exp-5 has been shown to function as an export receptor for small structured RNAs, among them miRNAs and adenoviral VA1-RNA (11, 23, 46). Nuclear export of dsRBD-containing proteins can also be mediated by Exp-5. Both ILF3 and mammalian Staufen 2 have been shown to be exported from the nucleus by an interaction with Exp-5 that is strongly stimulated by an RNA linker (12, 24).

The interaction between Exp-5 and the RNA-binding region of ADAR1 is dependent on RanGTP. dsRNA can stimulate the interaction between the RNA-binding region and Exp-5 but does not seem to be essential. The fact that dsRBDs treated with RNAse V1 can interact with Exp-5 argues for a direct but not RNA-mediated interaction of the two components. The stimulatory effect exerted by dsRNA might therefore be caused by conformational changes in the dsRBD induced by bound RNA.

Our results also suggest that TRN 1 and Exp-5 compete for binding to overlapping regions in ADAR1. As shown, the presence of Exp-5 prevents import of ADAR1 by TRN 1 in import assays. We attempted to clarify the binding characteristics of Exp-5 and TRN 1 on ADAR1 with in vitro pull-down assays. However, the different solubilities of the components involved precluded a conclusive analysis. Therefore, while it is likely that similar surfaces are recognized by both Exp-5 and TRN 1,

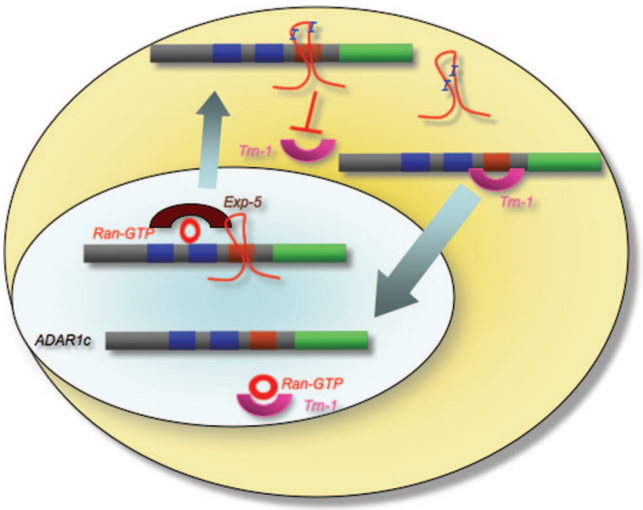

FIG. 7. Model of dsRNA-dependent nucleocytoplasmic shuttling of ADAR1. Cytoplasmic ADAR1 is bound by TRN 1 and imported to the nucleus, where TRN 1 is released by RanGTP. Within the nucleus, Exp-5 can interact with one or several dsRBDs in ADAR1, in a RanGTP-dependent manner, leading to nuclear export. This interaction can be stimulated by dsRNA. After nuclear export, RanGTP hydrolysis destabilizes the complex in the cytoplasm. However, interaction and thus reimport of RNA-bound ADAR1 are prevented by precluding an interaction of the third dsRBD with TRN 1 in the presence of dsRNA. Potentially, substrate RNAs can be exported with ADAR1 from the nucleus to the cytoplasm.

this point needs further clarification. In any case, in vivo Exp-5 and TRN 1 would bind at different sites of the nuclear membrane under the influence of different RanGTP concentrations and would therefore not compete directly for binding.

The dsRBD, an RNA-regulated nuclear transfer signal. Taken together, our data suggest a dual role for the third dsRBD in ADAR1 as both a nuclear import and export signal. The efficiency and directionality of the transport seem to be regulated by RNA. On the one hand, dsRNA interferes with the binding of TRN 1, while on the other, interaction with Exp-5 is enhanced. Therefore, nuclear import of ADAR1 is prevented in the RNA-bound state, while export is stimulated by RNA binding (Fig. 7). In fact, the FLIP experiments were in favor of this model, showing a slightly faster shuttling kinetics for wild-type ADAR1 than for mutant ADAR1 with impaired RNA binding. This is consistent with a stimulation of nuclear export by RNA binding. Nonetheless, the observed differences in FLIP assays were only minor, and RNA binding may thus be required only to control the directionality of transport. It remains to be determined, however, whether overexpression of cytoplasmic or nuclear substrate RNAs can influence the shuttling kinetics of wild-type but not mutant ADAR1.

If, as our data predict, RNA-associated nuclear ADAR1 is exported more efficiently by Exp-5, then the nature of such associated RNAs remains to be characterized. Several primiRNAs, for instance, have been reported to become edited within the nucleus. Some of these editing events lead to degradation of the RNA, which most likely occurs in the cytoplasm (30, 45). It is therefore likely that ADAR1 accompanies these substrates to the cytoplasm. Once in the cytoplasm, ADAR1 will be free for a new round of nuclear import only when the bound RNA has dissociated, therefore allowing binding of TRN 1 to the NLS in dsRBD3 (Fig. 7).

Our characterization of the dsRBD as an RNA-regulated nuclear import and export signal whose RNA-bound state determines the efficiency and direction of transport through the nuclear pore has potential implications for other dsRBD-containing proteins. In fact, several dsRBD proteins have been shown to be exported from the nucleus by Exp-5, including Jaz, ILF3, and Staufen 2 (7, 12, 24). However, nuclear import signals in these proteins have not been verified experimentally. It appears likely that the dsRBDs in these proteins also act as nuclear import signals. So far, we have tested several other dsRBDs for NLS activity. Among these, the first dsRBD of the *Xenopus* 4f1 protein, a homologue of human ILF3, proved to act as an NLS in transfection assays, also supporting the role of this dsRBD as a dual nuclear import and export signal. Also, several dsRBD proteins, in particular those involved in the RNAi pathway, are suspected to have functions in both the nucleus and the cytoplasm (27, 31). The shuttling mechanism described here may apply to some of them, possibly explaining their dual functions.

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