Transportin-mediated Nuclear Import of Heterogeneous Nuclear RNP Proteins

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Abstract. Heterogeneous nuclear ribonucleoprotein (hnRNP) A1 is an abundant nuclear protein that plays an important role in pre-mRNA processing and mRNA export from the nucleus. A1 shuttles rapidly between the nucleus and the cytoplasm, and a 38-amino acid domain, M9, serves as the bidirectional transport signal of A1. Recently, a 90-kD protein, transportin, was identified as the mediator of A1 nuclear import. In this study, we show that transportin mediates the nuclear import of additional hnRNP proteins, including hnRNP F. We have also isolated and sequenced a novel transportin homolog, transportin2, which may differ from transportin1 in its substrate specificity. Immunostaining shows that transportin1 is localized both in the cytoplasm and the nucleoplasm, and nuclear rim staining is also observed. The nuclear localization of A1 is dependent on ongoing RNA polymerase II transcription. Interestingly, a pyruvate kinase–M9 fusion, which normally localizes in the nucleus, also accumulates in the cytoplasm when RNA polymerase II is inhibited. Thus, M9 itself is a specific sensor for transcription-dependent nuclear transport. Transportin1–A1 complexes can be isolated from the cytoplasm and the nucleoplasm, but transportin1 is not detectable in hnRNP complexes. RanGTP causes dissociation of A1–transportin1 complexes in vitro. Thus, it is likely that after nuclear import, A1 dissociates from transportin1 by RanGTP and becomes incorporated into hnRNP complexes, where A1 functions in pre-mRNA processing.

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1. Abbreviations used in this paper: IBB, importin β binding domain; GST, glutathione-S-transferase; hn, heterogeneous nuclear; NLS, nuclear localization signal; NPC, nuclear pore complex; pol II, polymerase II.
yeast transportin (Pollard et al., 1996), which is the most closely related yeast protein to human transportin (35% identity; Nakiely et al., 1996). A recent report has described that a yeast protein, termed Kap104p, which is identical to yeast transportin, functions in the nuclear import of the mRNA-binding proteins, Nab2p and Nab4p, and in the reimport of exported nuclear mRNA-binding proteins (Aitchison et al., 1996).

In this study, we demonstrate that transportin is capable of interacting with hnRNP proteins other than A1 and that it mediates their nuclear import. We also describe a transportin homolog, termed transportin2, which likely has a distinct function as it has a different substrate specificity from the originally identified transportin, which hereinafter, we refer to as transportin1. By immunostaining, we show that transportin1 is localized both in the cytoplasm and the nucleoplasm and that nuclear rim staining can be observed, as is seen for importin β (Chi et al., 1995), suggesting that transportin1 interacts with NPCs during translocation. We found that nuclear localization of pyruvate kinase (PK) fused to M9, like A1, is transcription-dependent. Therefore, M9 is a transcription-dependent nuclear transport signal. We also demonstrate that transportin1-A1 complexes can be isolated from the nucleoplasm; however, no transportin1 is detectable in hnRNP complexes. Gel mobility shift assays show that addition of RanGTP causes dissociation of the transportin1-A1 complexes. Thus, we suggest that after nuclear import, A1 dissociates from transportin1 by RanGTP binding to transportin1 in the nucleoplasm and becomes incorporated into the hnRNP complexes where A1 functions in pre-mRNA metabolism (Choi et al., 1986; Mayeda and Krainer, 1992; Munroe and Dong, 1992; Caceres et al., 1994; Portman and Dreyfuss, 1994; Yang et al., 1994). We discuss the possible roles of transportins and hnRNP proteins in mRNA export.

Materials and Methods

Cell Culture, Labeling, and Cell Fractionation

HeLa S3 and HeLa monolayer-adapted clone JW36 cells were cultured at 37°C to subconfluent densities in DME supplemented with penicillin and streptomycin, and 10% calf serum. For the experiment shown in Figs. 4, A and B, cells were labeled with [35S]methionine (Amersham Corp., Arlington Heights, IL) at 20 μCi/ml for 20 h in DME containing one-tenth the normal methionine level and 5% calf serum. To prepare the cytoplasmic and nucleoplasmic fractions, cells were resuspended in RSB100, and inhibitors and competitors of importins α and β have no effect on M9-mediated import (Pollard et al., 1996). Thus, the transportin-mediated nuclear import pathway is distinct from the importin-mediated pathway. However, sequence comparison reveals that transportin is distantly related (24% identity) to human importin β (Pollard et al., 1996). In addition, the transportin-mediated protein import is inhibited by RanQ69L (Nakiely et al., 1996), a known inhibitor of classical NLS-bearing protein import (Melchior et al., 1995; Marshall et al., 1996; Palacios et al., 1996), suggesting that Ran, or a Ran-like molecule, is required for transportin-mediated protein import, as is the case for importin-mediated import. As described previously, there is a transportin homolog in Saccharomyces cerevisiae.
in vitro transcription–translation of plasmids His-transportin, pCRimpβ (Pollard et al., 1996), and His-transportin2, respectively, using a TnT kit (Promega Biotech, Madison, WI) in rabbit reticulocyte lysate in the presence of [35S]methionine (Amersham Corp.), and exposed to X-ray film.

The cDNA for hnRNP F (Matunis et al., 1994; containing PCR-engineered BamHI and PstI sites just outside the initiation and termination codons, respectively) was excised from pG8T9-F by digestion with PstI, mung bean nuclease, and BamHI, and subcloned into pGEX-5X-1 (Pharmacia Fine Chemicals, Piscataway, NJ) at the BamHI and Smal sites. The plasmid was transformed and expressed in BL21(DE3) cells, and the glutathione-S-transferase (GST)–F fusion protein was purified according to the manufacturer’s instructions. The BstBI/PvuII fragment of hnRNP C2 from pHCC2 (Burd and Dreyfuss, 1994) was subcloned into the same sites in hnRNP C1 in pCDNA3.1 (Nakielny and Dreyfuss, 1996). The entire hnRNP C2 vector in this vector was then excised with EcoRI and Xhol and subcloned into pGEX-5X-3 (Pharmacia Fine Chemicals). This plasmid was likewise transformed and expressed in BL21(DE3) cells, and the GST–C2 fusion was purified as described above.

### Protein-Binding Assays

Purified wild-type GST-M9 or the import-defective GST-M9 mutant (G734 to A; 5 μg each) were incubated with 30 μl of glutathione-Sepharose (Pharmacia Fine Chemicals) in 500 μl of binding buffer (50 mM Tris–HCl, 400 mM NaCl, 5 mM Mg(OAc)2, 2 μg/ml leupeptin, 2 μg/ml pepstatin, and 0.5% aprotinin, pH 7.5). After incubation for at least 1 h at 4°C, the resin was washed with binding buffer, and the cytoplasmic fraction of HeLa cells was added. After incubation for 3 h at 4°C, the resin was washed with binding buffer and the bound fraction was eluted by boiling in SDS-PAGE sample buffer, analyzed by SDS-PAGE, and visualized by autoradiography.

### Nuclear Import Assays

Nuclear import reactions were performed as described (Adam et al., 1990). The transport substrates were added at a concentration of 100 μg/ml. After the import reactions, the nuclei were fixed and processed for immunostaining (see Immunofluorescence Microscopy). Immunoprecipitations were carried out in the presence of EmpigenBB at 1%, 1 mM EDTA, and 0.1 mM DTT as described (Choi and Dreyfuss, 1984) from either [35S]methionine–labeled HeLa cell lysate or rabbit reticulocyte lysate in which transportin1 and 2 were produced by in vitro transcription–translation using a TnT kit (Promega Biotech) in the presence of [35S]methionine.

The preparation of the monoclonal antibodies 4F4 (anti-hnRNP C) and 4B10 (anti-hnRNP A1) were described previously (Choi and Dreyfuss, 1984; Piñol-Roma et al., 1988). For the experiment shown in Fig. 8 B, immunoprecipitations were carried out in the presence of EmpigenBB at 1% from rabbit reticulocyte lysate in which myc-PK, myc-PK-M9, and myc-A1 were produced by in vitro transcription–translation of plasmids (Michael et al., 1995b; Siomi and Dreyfuss, 1995) using a TnT kit (Promega) in the presence of [35S]methionine.

### Full Length Transportin2 Isolation

In the course of isolation of full length transportin1 cDNA described previously (Pollard et al., 1996), we obtained several partial clones (transportin2) with high similarity to transportin1. One longer clone among them was then employed as a probe to screen a ZAP II HeLa cDNA library (Stratagene Corp., La Jolla, CA) to obtain the full length cDNA of transportin2. Several positive clones were obtained and sequenced. Composite full length cDNA of transportin2 was inserted into pET28a vector (Novagen, Madison, WI) to construct the expression plasmid, His-transportin2. The DNA sequence of transportin2 is available in the EMBL/Genbank/DDBJ database under accession number AF019039.

### Preparation of Monoclonal Antibodies

The anti-transportin1 monoclonal antibody D45 was obtained by immunization of a BALB/c mouse with recombinant His-tagged transportin1 (Pollard et al., 1996) purified from E. coli. To demonstrate the specificity of D45, immunoprecipitation was carried out in the presence of the ionic detergent EmpigenBB at 1%, 1 mM EDTA, and 0.1 mM DTT as described (Choi and Dreyfuss, 1984) from either [35S]methionine–labeled...
of RNA (A1 winner), and 1 U/μl of RNasin (Promega Biotech). 5% native polyacrylamide gels were used to analyze the complexes.

Results

Transportin1 Mediates Import of Other hnRNP Proteins in Addition to A1

Although M9 sequence is not found in other known proteins, we considered the possibility that transportin1 may facilitate the import of other hnRNPs. To identify new nuclear import substrates, we isolated an enriched hnRNP population from HeLa cell nucleoplasm by single-stranded DNA-agarose chromatography. The partially purified hnRNP preparation was resolved by SDS-PAGE and transferred to nitrocellulose. The immobilized proteins were then probed with either 35S-labeled transportin1 or importin β that were synthesized in rabbit reticulocyte lysate. Several proteins showed strong interaction with transportin1, but not with importin β (Fig. 1). In addition to A1, other candidate proteins in the profile with transportin1 included the hnRNP B, D, E, and F proteins (Dreyfuss et al., 1993). The protein band labeled “X” is an artifact that appears even after probing with an unprogrammed control reticulocyte lysate (data not shown). When the protein preparation was alternatively subjected to two-dimensional Nephge/SDS-PAGE, followed by electrotransfer and probing with labeled transportin1, one of the signals on the two-dimensional blot corresponded to a protein of molecular weight and isoelectric point coincident with hnRNP F (data not shown; Matunis et al., 1994). The possibility that hnRNP F could interact with transportin1 in vitro was investigated by expressing and purifying hnRNP F as the recombinant fusion protein GST-F and testing whether nitrocellulose-bound GST-F could interact with 35S-labeled transportin1. In addition, we also tested hnRNP C2 fused to GST, since one of the strongly interacting proteins in Fig. 1 corresponds in size to hnRNP C proteins (41–43 kD). Fig. 2A shows that under conditions where hnRNP C2 (Burd et al., 1989) fused to GST is incapable of binding transportin1, there is a strong interaction between transportin1 and GST-F and between transportin1 and GST-M9. To test whether the hnRNP F-transportin1 interaction could be competed by an M9-containing fragment of hnRNP A1, we carried out a protein-binding assay, in which GST-F was bound to glutathione-Sepharose beads. The complex was incubated with 35S-labeled transportin1 in the presence or absence of the competitor zz-M3 (Pollard et al., 1996), a fusion protein containing the zz peptide, the interaction domain of protein A with IgG, fused to the hnRNP A1 M3 fragment, which includes the M9 domain plus an additional 32 amino acids NH2-terminal to M9 and 15 amino acids COOH-terminal to M9 (Siomi and Dreyfuss, 1995). A 10-fold molar excess was sufficient to nearly completely block the binding of transportin1 to either GST-M9 or to GST-F (Fig. 2B).

The ability of transportin1 to mediate nuclear import of hnRNP F was assessed in an in vitro import assay system (Adam et al., 1990), using digitonin-permeabilized HeLa cells. In the absence of HeLa cytosol, GST-F is unable to be imported into the nucleus, whereas the addition of HeLa S100 cytosol facilitates protein import (data not shown). In the absence of HeLa cytosol, GST-M9 can be imported with the addition of exogenous transportin1. Fig. 2C shows that GST-F can also be imported in permeabilized HeLa cells upon the addition of exogenous transportin1, showing directly that transportin1 mediates hnRNP F import.

Transportin2, a transportin1 Relative, Has Different Substrate Binding

In the course of isolating full length transportin1 cDNA (Pollard et al., 1996), we obtained several partial clones that had high similarity to the originally identified transportin1. One of these clones showed 84% amino acid sequence identity to transportin1. We termed this new 894-amino acid homolog transportin2. The amino acid sequence alignment of transportin2 and transportin1 is shown in Fig. 3A. The two proteins are highly similar over their entire length. Two notable exceptions include differences in the acidic stretch found in the middle of transportin1 (586DEGDIEEDDDDEIDDDDD606) and transportin2 (586EAERPDGSEDADDDDDDD626), and most notably, an extra sequence near the COOH end of transportin2 (626GRLTSPSAID774) which is not found in transportin1. Thus, while yeast contains only one transportin gene (yTRN/Kap104p; Aitchison et al., 1996; Nakielny et al., 1996) there are at least two transportin homologs in humans. Far Western blotting experiments showed that transportin2 did not bind any of the proteins in the ss-DNA-binding protein fraction, whereas transportin1, under the same conditions, bound avidly to several of them (Fig. 3B). The COOH half of transportin1 (amino acids 518 to the end of the protein) is sufficient for interaction with the M9 domain of A1 (Pollard et al., 1996), and the extra sequence, located in this region of transportin2 corresponding to the M9-interacting domain of transportin1, likely modifies the interaction preference and/or strength.
of transportin2 interaction with these proteins. The identity of the nuclear import substrates of transportin2, if any, is as yet unknown.

**D45, a Monoclonal Antibody Specific for Transportin1**

To further characterize transportin1 and its interaction with hnRNP proteins, we generated monoclonal antibodies to it by immunizing mice with purified recombinant 6His-tag transportin1. Several monoclonal antibodies were obtained, and one of these, termed D45, was further characterized in detail. By immunoprecipitation in the presence of the ionic detergent EmpigenBB, D45 specifically immunoprecipitated transportin1 but not importin β, to which transportin1 is distant related (Fig. 4A). The specificity of D45 was further demonstrated as D45 immunoprecipitated, from total HeLa cell extract, a single 90-kD protein that comigrated by SDS-PAGE with in vitro-translated transportin1 (Fig. 4A). Similar immunoprecipitation was also performed using in vitro-translated transportin2, and although the amino acid sequence of this protein is highly similar to that of transportin1, D45 did not show detectable cross-reactivity with transportin2 (Fig. 4B). Deletion experiments suggest that the epitope of D45 is located within the second quarter of transportin1 (data not shown). The lack of cross-reactivity of D45 with transportin2 suggests that the acidic stretch region showing at least similarity between transportins 1 and 2 (see Fig. 3A) may be the epitope of D45, and the acidic region might determine, in part, some functional difference between transportins 1 and 2.

Immunoblotting using D45 on HeLa cytoplasm incubated with either wild-type GST-M9 or the import defective GST-M9 mutant (G274 to A; Michael et al., 1995) at 400 mM NaCl, showed a single 90-kD protein bound to GST-M9 but not to the GST-M9 mutant (Fig. 5A, Coomassie) that reacted with D45 (Fig. 5A, TRN1 blot). This confirmed the specific binding of transportin1 to M9. Immunoblotting with D45 on lysates from several vertebrate organisms was carried out (Fig. 5B). D45 cross-reacts with protein bands of similar mobility to human transportin1 in monkey and rabbit but not in quail and frog.

**Subcellular Localization of Transportin1**

Laser confocal immunofluorescence microscopy using D45 was performed to determine the subcellular localization of transportin1. For comparison, antibodies to hnRNP A1 (4B10; Choi and Dreyfuss, 1984) and importin β (3E9; Chi et al., 1995) were also used. As shown in Fig. 5C, transportin1 is found not only throughout the cytoplasm, but also in the nucleoplasm. Intense nuclear rim staining was also observed, although it is not as striking as that for importin β (Chi et al., 1995). The nuclear rim staining suggests binding to the NPCs, as expected for nuclear transport factors. We also expressed transportin1 as a fusion protein with a myc epitope tag by transfection in HeLa cells and observed that the localization of the myc-tagged transportin1 in transfected cells agreed with that seen by the antibody staining with D45 (data not shown).

**M9 is a Transcription-dependent Nuclear Localization Signal**

Previous studies have shown that the nuclear localization of hnRNP A1 is dependent on RNA pol II transcription (Piñol-Roma and Dreyfuss, 1991, 1992). To test whether M9 is the region in A1 that confers the transcription sensitivity to A1 nuclear localization, HeLa cells were transfected with full length A1 or PK fused to M9 and then the transfected cells were treated with a pol II inhibitor, actinomycin D for 4 h. As expected, the full length A1 overexpressed in HeLa cells behaved like the endogenous A1; in contrast, PK-M9, which was localized in the nucleus in untreated cells as full length A1, accumulated entirely in the cytoplasm and was apparently absent from the nucleus in cells treated with the pol II inhibitor (Fig. 6). In this experiment, the hnRNP C proteins were detected exclusively in the nucleus (data not shown). This indicates that M9 itself...
is the specific sensor of A1 for transcription-dependent nuclear transport. The intracellular distribution of transportin1 was not affected with the pol II inhibitor under the same conditions (data not shown). Therefore, it is likely that the absence of pol II transcription impairs the interaction between M9 and transportin1 in the cytoplasm, resulting in the accumulation of A1 in the cytoplasm.

**Transportin1 Exists as a Complex with A1 in the Nucleoplasm, But Not in hnRNP Complexes**

HnRNP A1-transportin1 complexes must exist in the cytoplasm because A1 cannot be imported into the nucleus without interacting with transportin1. The question we raised then, was whether A1-transportin1 complexes also exist in the nucleoplasm in living cells. To address this, we carried out immunoprecipitations from the nucleoplasm using 4B10 (anti-A1) and 4F4 (anti-C), and the presence of transportin1 in the immunoprecipitates was examined by Western blotting using D45. Under the conditions employed in this immunoprecipitation study, hnRNP complexes consisting of >20 different hnRNP proteins on pre-mRNAs (Dreyfuss et al., 1993) can be isolated. As expected, transportin1 was in the 4B10 immunoprecipitate, demonstrating that transportin1 is still associated with A1 in the nucleoplasm after translocation through NPCs (Fig. 7 A).

However, in the 4F4 immunoprecipitate, no transportin1 was detectable, although the immunoprecipitation of hnRNP complexes with 4F4 was efficient, as assessed by coimmunoprecipitation of A1. There are two possible explanations for this observation. First, there may be subsets of hnRNP complexes containing transportin1 that are immunoprecipitable with 4B10, but not with 4F4. Second, transportin1 may not be a component of hnRNP complexes in the nucleoplasm.

To determine which of these scenarios is more likely, similar immunoprecipitations were carried out from the nucleoplasm after treatment with RNase, which causes dissociation of all hnRNP complexes. As shown in Fig. 7 B, transportin1 was still in the 4B10 immunoprecipitate, whereas hnRNP C1 protein was no longer detected by Western blotting (demonstrating the efficiency of the RNase digestion). We therefore conclude that transportin1 is not associated with hnRNP complexes, or that only very small amounts are present.

**M9 Is Not Accessible to Transportin1 while A1 Is in hnRNP Complexes**

Several monoclonal anti-A1 antibodies have been produced in our lab (Piñol-Roma, S., and G. Dreyfuss, unpublished observations). Interestingly, when immunoprecipit-
Figure 4. Specificity of the monoclonal antibody for transportin1, D45. (A) Transportin1 (TRN1) and importin β (Impβ) were transcribed–translated in vitro in the presence of [35S]methionine (translation). Immunoprecipitations were carried out with D45 and SP2/0 (as a control) in the presence of the ionic detergent EmpigenBB, and the bound fraction of the translated products was analyzed by SDS-PAGE and visualized by fluorography (immunoprecipitation). Products of transcription–translation reaction are shown as translation. Additional immunoprecipitation was carried out from total HeLa extract labeled with [35S]methionine under the same conditions. Note that D45 reacts specifically with transportin1 and does not cross-react to importin β. The positions of molecular mass markers are indicated on the left. (B) Immunoprecipitation of transportins 1 and 2 with D45. Immunoprecipitation was carried out with D45 using transportins 1 (TRN1) and 2 (TRN2) transcribed–translated in vitro in the presence of [35S]methionine (translation) as described above. D45 does not cross-react to transportin2.

Figure 5. (A) M9-containing protein specifically interacts with transportin1 among all the cytoplasmic proteins from HeLa cells. GST-M9 or the import-defective GST-M9 mutant (G274 to A; Michael et al., 1995b) on glutathione-Sepharose (both indicated by GST-) was incubated with the cytoplasmic fraction from HeLa cells in the presence of 400 mM NaCl. The total HeLa cytoplasmic fraction and the bound fraction to the GST-fusion proteins were analyzed by SDS-PAGE and either visualized by Coomassie staining (Coomassie blue) or by immunoblotting with D45 (TRN1 blot). Transportin1 specifically interacting with GST-M9 but not with the mutant (GST-M9 mut) is indicated by TRN1 with an arrow. GST- indicates the GST-fusion proteins bound on glutathione-Sepharose beads. The positions of molecular mass markers (MW) are indicated on the left. (B) Zoo blot analysis with D45. Approximately equal amounts of total proteins from HeLa (Human), COS (Monkey), QT-6 (Quail), and XL177 (Xenopus) cells and rabbit reticulocyte lysate (Rabbit) were separated by SDS-PAGE, transferred to a nitrocellulose membrane, and probed with D45. The immunoblot signals were visualized with the ECL kit (Amersham). D45 cross-reacts to protein bands of similar mobility to human transportin1 in monkey and rabbit (indicated by TRN1 with an arrow), but not in quail and frog. (C) Subcellular localization of transportin1 in HeLa cells. HeLa cells grown on glass coverslips were fixed with 2% formaldehyde, permeabilized with 0.1% Triton X-100, and incubated with either anti-hnRNP A1 protein, 4B10 (Choi and Dreyfuss, 1984; Piñol-Roma et al., 1988), anti-importin β, 3E9 (Chi et al., 1995), or D45. The primary antibodies were recognized with FITC-conjugated goat anti–mouse antibodies, and the confocal images of the protein staining were analyzed on a Leica confocal microscope. Transportin1 is localized both in the cytoplasm and the nucleoplasm and is also accumulated in the nuclear rim as seen for importin β (Chi et al., 1995).
tions were carried out with a different anti-A1 antibody, 9H10, transportin1 was not detected in immunoprecipitates from either the cytoplasm or the nucleoplasm. However, in contrast, 4B10 coimmunoprecipitated transportin1 along with A1 from both compartments under the same conditions (Fig. 8 A). This difference may be due to the different epitopes recognized by 4B10 and 9H10, and we therefore performed epitope-mapping experiments. In the presence of the ionic detergent EmpigenBB, immunoprecipitations were carried out with 4B10 and 9H10 from rabbit reticulocyte lysate in which myc-tagged PK, myc-PK-M9, and myc full length A1 (Siomi and Dreyfuss, 1995; Michael et al., 1995b) were translated in the presence of [35S]methionine. The data shown in Fig. 8 B clearly demonstrate that the epitope of 9H10 is located within the M9 region of A1, providing an explanation for the inability of 9H10 to immunoprecipitate A1 that is bound to transportin1. When immunoprecipitation of hnRNP complexes was carried out from HeLa nucleoplasm using 9H10, along with 4B10 and 4F4 for comparison, 9H10 did not immunoprecipitate hnRNP complexes (Fig. 8 C). We conclude that M9, the interaction domain of A1 with transportin1, is not accessible to transportin1 once A1 is assembled into hnRNP complexes.

**RanGTP Dissociates A1-Transportin1 Complexes**

RanGTP causes the NLS cargo-importin α/β complexes to dissociate at the nucleoplasmic side of the NPCs (Rexach and Blobel, 1995; Görlich et al., 1996), and since transportin1 interacts with RanGTP (Nakielny, S., F.R. Bischoff, and G. Dreyfuss, manuscript in preparation), it was of interest to test whether RanGTP also dissociates transportin1-A1 complexes. To address this, we devised the following gel shift assay. A1 and an RNA probe (A1 winner; UAUGAUAGGGACUUAGGGUG, 32P-labeled; Burd and Dreyfuss, 1994) were incubated in the presence of either transportin1 or bovine serum albumin (BSA), and the formation of complexes was analyzed by a gel mobility shift assay (Kataoka et al., 1995). As shown in Fig. 9 A, addition of BSA to A1-RNA complexes had no effect (lanes 9–11); in contrast, addition of transportin1 resulted in the formation of a new complex of lower mobility (Fig. 9 A, lanes 3–5), as expected if transportin1 could interact with A1 while it binds RNA. Transportin1 did not detectably bind to RNA on its own (lanes 6–8).

To examine the effect of RanGTP on A1-transportin1 complexes, A1 and RNA (A1 winner) were pre-incubated on ice in the presence of transportin1, and after addition of RanGTP, the complexes were analyzed. Fig. 9 B shows that addition of RanGTP resulted in the disappearance of the transportin1-A1–RNA complexes, while A1-RNA complexes remained intact (lanes 5–7). Thus, the interaction of RanGTP with the transportin1-A1–RNA complexes, most likely through transportin1, causes their dissociation. RanQ69L (GTP form) also dissociated transportin1 from A1 (Fig. 9 B, lanes 7–9), whereas addition of RanGDP had no effect (Fig. 9 B, lanes 11–13).

**Discussion**

Transportin1 interacts directly and specifically with M9, the bidirectional transport signal of the nuclear shuttling protein, hnRNP A1 (Michael et al., 1995b; Siomi and Dreyfuss, 1995) and mediates the nuclear import of hnRNP A1 (Nakielny et al., 1996; Pollard et al., 1996). In this study, we have shown that transportin1 is also capable of interacting with additional hnRNP proteins, such as...
hnRNP F (Matunis et al., 1994) and mediates their nuclear import in an in vitro import assay. The interaction of hnRNP F protein with transportin1 is competed by the M3 region of A1 (Siomi and Dreyfuss, 1995; Pollard et al., 1996), suggesting that the same region of transportin1 (amino acids 518 to the end of the protein; Pollard et al., 1996) interacts with hnRNP A1 and F proteins. We have searched for an M9-like domain in the hnRNP F sequence, but no obvious sequence similarity was revealed. However, we note that hnRNP F contains a region, between the second and the third RNA-binding domains that is rich in Gly, Ser, Asn, and Tyr residues (Matunis et al., 1994), an amino acid composition similar to that of A1-M9.

Therefore, transportin1 likely recognizes its import substrates by secondary and/or tertiary structural features rather than by primary sequences. Transportin1 is distant to human importin β (24% identity; Nakielny et al., 1996). Nevertheless, transportin1 has a few characteristics that distinguish it from importin β in terms of interacting with its import substrates: (a) transportin1 does not require adaptor proteins to interact with its import substrates, whereas importin β interacts with its import substrates (the classical NLS-bearing proteins) via importin α (Görlich et al., 1995a; Imamoto et al., 1995a); and, related to this, (b) transportin1 recognizes a wider range of sequences on its import substrates, whereas importin β binds strictly to the IBB of importin α (Görlich et al., 1995a; Weis et al., 1996).

Although transportin2 has very high sequence similarity to transportin1, it does not bind any of the ssDNA-binding proteins on a far Western blot under the same conditions at which transportin1 produced strong signals. One of the most obvious differences between these two protein sequences is a small peptide present near the COOH end of transportin2 located within the region corresponding to the M9-interacting domain of transportin1 (amino acids 518 to the end of the protein; Pollard et al., 1996). Therefore, it is likely that the presence or absence of this mini-exon-like sequence modifies the interaction of transportins1 and 2 with import substrates. The other notable sequence difference between transportins1 and 2 is the acidic stretches located within the second quarter of both proteins. Importin β contains such an acidic stretch, and this sequence is part of its Ran/NPC-binding domain (Chi et al., 1996; Kutay et al., 1997). In Ran also, an acidic stretch near its COOH end is required for the high-affinity binding of RanGTP to RanBP1 (Lounsbury et al., 1994; Richards et al., 1995; Bischoff et al., 1995; Ren et al., 1995) and

Figure 8. M9 is not accessible in hnRNP complexes. (A) Cytoplasmic (C) and nucleoplasmic (N) fractions were prepared from HeLa cells, and immunoprecipitations were carried out with 4B10 and 9H10 (anti-hnRNP A1 antibodies). Note that 9H10 can immunoprecipitate A1 (hnRNP A1); however, transportin1 (TRN1) is not detectable in the 9H10 immunoprecipitates from either compartment. (B) Epitope mapping of 4B10 and 9H10. The in vitro transcription-translation was carried out for PK, PK-M9, and full length hnRNP A1 (A1) in the presence of [35S]methionine (translation), and immunoprecipitation was performed using 4B10 and 9H10 in the presence of EmigjenBB. The bound fraction was analyzed by SDS-PAGE and visualized by fluorography. Both antibodies are capable of immunoprecipitating full length A1, but only 9H10 can immunoprecipitate PK-M9, indicating that the epitope of 9H10 is within the M9 region of A1. (C) 9H10 does not immunoprecipitate hnRNP C1/C2 proteins. Immunoprecipitations were carried out from the nucleoplasmic fraction of HeLa cells labeled with [35S]methionine using 4B10, 4F4, and 9H10. After immunoprecipitation, all proteins were analyzed by SDS-PAGE and visualized by fluorography. Proteins corresponding to hnRNP C1/C2 proteins are not observed in the 9H10 immunoprecipitate, indicating that 9H10 cannot immunoprecipitate hnRNP complexes.
to affect the role of RanBP1 as a costimulator of RanGAP (Becker et al., 1995; Bischoff et al., 1995; Richards et al., 1995). Therefore, it is possible that transportins 1 and 2 have distinct functions in protein transport through NPCs, and the acidic regions may play important roles in distinguishing their functions from each other.

Here we provide evidence that A1-transportin 1 complexes dissociate by RanGTP binding to transportin 1. In the nucleus, presumably after its dissociation from transportin 1, A1 becomes incorporated into hnRNP complexes, where it functions in pre-mRNA processing. Together with the transportin 1 import inhibition data with RanQ69L (Nakielny et al., 1996), this observation indicates that Ran and GTP hydrolysis function similarly in importin-mediated and transportin 1-mediated nuclear import. However, the dissociation of A1 is not complete, since we could isolate A1-transportin 1 complexes from the nucleoplasmic fraction. We also observed by immunoprecipitation experiments with D45 that not all transportin 1 appears to be associated with import substrates in the nucleoplasm (data not shown), indicating that some transportin 1 remains free in the nucleoplasm after dissociating from its cargo. These observations agree well with the immunostaining data with D45, which show that transportin 1 is localized in the nucleoplasm to a greater extent than importin β. This suggests that transportin 1 may have roles in the nucleus in addition to its role in importing hnRNP proteins from the cytoplasm. They are, however, presently not yet known. The capacity of RanGTP to completely dissociate transportin 1 in vitro while transportin 1-A1 complexes are found in the nucleus suggests that other factors, such as Ran-binding proteins, may stabilize these complexes in the nucleus. Alternatively, RanGTP may not be homogeneously distributed in the nucleus.

HnRNP A1 shuttles rapidly between the nucleus and the cytoplasm (Piolot-Roma and Dreyfuss, 1992). A1 is bound, at least initially, to poly(A) + RNA while in the cytoplasm, and it has been recently shown by immunoelectron microscopy that mRNA in transit through the NPC to the cytoplasm is indeed associated with hnRNP A1/A2-type proteins (Mehlin et al., 1992; Mehlin and Daneholt, 1993; Visa et al., 1996; Daneholt, 1997). Therefore, A1 is likely to play an important role in the export of mRNAs from the nucleus. Recent nuclear microinjection experiments provide additional direct evidence for this suggestion (Izaurralde et al., 1997). The M9 domain of A1 has been shown to serve as the bidirectional transport signal of A1 (Michael et al., 1995b; Siomi and Dreyfuss, 1995), and its NLS and nuclear export signal have not been separable so far (Michael et al., 1995b). The factors that interact with M9 and mediate the import and export of A1 may be the same, and in exhaustive screens, transportin 1 has been the only specific M9-binding factor found. Thus, although there is no detectable transportin 1 with bulk hnRNP complexes and M9 is not accessible to both transportin 1 and 9H10, an anti-M9 monoclonal antibody, it is possible that transportin 1 (or a close relative, such as transportin 2) is involved in mRNA export. For example, if transportin 1 binds to hnRNP complexes after splicing but immediately before their association with NPCs, this fraction may be too small to detect, and it would not be contained in the soluble nucleoplasmic fraction from which we can immunoprecipitate hnRNP complexes; NPCs fractionate with the insoluble “chromatin/nucleolar” pellet. It is also possible that, in contrast to the 1:1 stoichiometry (transportin 1: A1) that is required for A1 nuclear import, a much smaller
amount of transportin1 (e.g., one transportin1 molecule per hundreds of A1 molecules) is sufficient to direct hnRNP complexes to the NPCs and mediate their export, and this may be below our level of detection.

Finally, the difference in the regulation of the importin- and transportin-mediated nuclear import pathways provides a framework for thinking about the need for these separable pathways. Nuclear import of some hnRNP proteins, represented by A1, is dependent on pol II transcription. In this context, it is interesting that excess free A1 microinjected into Xenopus oocyte nuclei specifically inhibits mRNA export (Izaurralde et al., 1997). It therefore appears that the reason for reducing the amount of A1 in the nucleus when pol II activity is reduced is to prevent excess A1 from competing with mRNA export. It is also possible that A1 in excess of RNA-binding sites is deleterious to the nucleus because it may be insoluble. Therefore, substrates of transportin1, such as A1, needed to evolve their own nuclear import pathway different from the importin-mediated pathway. The accumulation of PK-M9 in the cytoplasm in cells treated with a pol II inhibitor (actinomycin D) indicates that M9 is a transcription-dependent nuclear transport signal. The accumulation of M9-bearing proteins in the cytoplasm in the presence of actinomycin D is probably a result of lack of interaction of M9 with transportin1 in the absence of pol II transcription, since the intracellular distribution of transportin1 itself is transcription independent (data not shown). PK-M9 accumulates in the cytoplasm to a much greater extent than full length A1 in response to actinomycin D treatment. A1 has many functions and interactions in the nucleus while it binds pre-mRNA along with all other hnRNP proteins. Since M9 lacks the RNA-binding domains and an RGG box, which A1 contains, PK-M9 has fewer interactions with other nuclear components. This is probably why M9 accumulates in the cytoplasm to a much greater extent than A1 in the presence of actinomycin D. Transportin1 isolated from cells treated with actinomycin D is still capable of interacting with GST-M9 fusion protein on glutathione-Sepharose beads as well as that from untreated cells (data not shown). Future experiments will examine possible modifications, such as phosphorylation, that may take place on M9 and, in turn, prevent its interaction with transportin1 in transcriptionally inhibited cells.

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