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The Nucleoporin Nup153 Plays a Critical Role in Multiple Types of Nuclear Export

Katharine S. Ullman,*† Sundeep Shah, Maureen A. Powers,‡ and Douglass J. Forbes

Department of Biology, University of California at San Diego, La Jolla, California 92093-0347

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The fundamental process of nucleocytoplasmic transport takes place through the nuclear pore. Peripheral pore structures are presumably poised to interact with transport receptors and their cargo as these receptor complexes first encounter the pore. One such peripheral structure likely to play an important role in nuclear export is the basket structure located on the nuclear side of the pore. At present, Nup153 is the only nucleoporin known to localize to the surface of this basket, suggesting that Nup153 is potentially one of the first pore components an RNA or protein encounters during export. In this study, anti-Nup153 antibodies were used to probe the role of Nup153 in nuclear export in *Xenopus* oocytes. We found that Nup153 antibodies block three major classes of RNA export, that of snRNA, mRNA, and 5S rRNA. Nup153 antibodies also block the NES protein export pathway, specifically the export of the HIV Rev protein, as well as Rev-dependent RNA export. Not all export was blocked; Nup153 antibodies did not impede the export of tRNA or the recycling of importin β to the cytoplasm. The specific antibodies used here also did not affect nuclear import, whether mediated by importin α/β or by transportin. Overall, the results indicate that Nup153 is crucial to multiple classes of RNA and protein export, being involved at a vital juncture point in their export pathways. This juncture point appears to be one that is bypassed by tRNA during its export. We asked whether a physical interaction between RNA and Nup153 could be observed, using homoribopolymers as sequence-independent probes for interaction. Nup153, unlike four other nucleoporins including Nup98, associated strongly with poly(G) and significantly with poly(U). Thus, Nup153 is unique among the nucleoporins tested in its ability to interact with RNA and must do so either directly or indirectly through an adaptor protein. These results suggest a unique mechanistic role for Nup153 in the export of multiple cargos.

INTRODUCTION

Compartmentalization of genomic DNA within the nucleus provides a unique environment in which replication and expression of the genome can be tightly regulated. In turn, nucleocytoplasmic transport is critical to these fundamental processes. A major challenge in the field has been to determine how nuclear pores accomplish the complicated task of selective, bidirectional trafficking. Initial steps in understanding nucleocytoplasmic transport have involved characterization of the molecular nature of transport cargo traversing the pore. Much progress has been made not only in defining signals that direct nucleocytoplasmic traffic but also in identifying cognate soluble receptors that ferry cargo to and through the pore (for review, see Corbett and Silver, 1997; Izaurralde and Adam, 1998). The first such receptor to be identified was importin α (also referred to as NLS receptor, karyopherin α, PTAC58, and hSRP1), a protein that binds to the clas-

* Corresponding author. E-mail address: katharine.ullman@hci.utah.edu.
Present addresses: † Department of Oncological Sciences, Huntsman Cancer Institute, University of Utah, Salt Lake City, UT 84108; ‡ Department of Cell Biology, Emory University, Atlanta, GA 30322.

sical basic nuclear localization signal (NLS) (Adam and Gerace, 1991; Görlich *et al.*, 1994; Imamoto *et al.*, 1995; Moroianu *et al.*, 1995; Weis *et al.*, 1995). Importin α directs import as part of a heterodimer; its partner importin β (p97, karyopherin β, and PTAC97) is primarily responsible for mediating contact with nucleoporins during transit of the receptor complex through the pore (Chi *et al.*, 1995; Görlich *et al.*, 1995; Radu *et al.*, 1995a). Importin β is a member of a large superfamily of related proteins (Fornerod *et al.*, 1997b; Görlich *et al.*, 1997). Recent results have implicated an increasing number of members of this family as specialized import receptors (Aitchison *et al.*, 1996; Pollard *et al.*, 1996; Pemberton *et al.*, 1997; Rosenblum *et al.*, 1997; Rout *et al.*, 1997; Ferrigno *et al.*, 1998; Jakel and Görlich, 1998; Senger *et al.*, 1998). In most cases these importin β-like receptor proteins have been found to bind to specific transport substrates directly rather than in conjunction with a partner protein.

Although numerous classes of RNA and a number of proteins are exported from the nucleus, less is known about the specific signals and cognate receptors that direct their export. One important step forward was the identification of a short, leucine-rich amino acid sequence termed the nuclear export signal or NES that, like the NLS, mediates interaction with a soluble receptor, in this case exportin 1/Crm1 (Fornerod *et al.*, 1997a interaction with a soluble receptor,; Fukuda *et al.*, 1997; Neville *et al.*, 1997; Ossareh-Nazari *et al.*, 1997; Stade *et al.*, 1997; Ullman *et al.*, 1997). In fact, this export receptor was found to be a member of the importin β superfamily. NESs were identified originally in the human immunodeficiency virus (HIV) protein Rev and in the cellular protein PKI (protein kinase A inhibitor; Fischer *et al.*, 1995; Wen *et al.*, 1995). Competition analysis soon made it apparent, however, that an NES signal is involved in many types of nuclear export including 5S RNA, U snRNA, and mRNA export (Fischer *et al.*, 1995; Pasquinelli *et al.*, 1997b). This implies that the NES receptor exportin 1 is similarly multifunctional; however, the role of exportin 1 in the export of all of these cargo has not yet been entirely elucidated.

tRNA export, although known to be an active export process (Zasloff, 1983; Jarmolowski *et al.*, 1994), shows no dependence on an NES signal in competition studies (Pasquinelli *et al.*, 1997b). Recently, a soluble receptor termed exportin-t was identified and is proposed to guide tRNA from the nucleus to the cytoplasm (Arts *et al.*, 1998; Hellmuth *et al.*, 1998; Kutay *et al.*, 1998). The protein sequence of exportin-t reveals that it too is a member of the importin β superfamily. Interestingly, rather than recognizing a peptide signal sequence, exportin-t interacts directly with tRNA, illustrating another variation of cargo recognition in this family.

In summary, evolution appears to have elegantly created a family of specialized receptors that fulfill myriad transport functions. Although each member has unique specificity, a common defining feature shared by all members is a binding domain for the small GTPase Ran, an important accessory factor for nuclear transport. The distinct roles of Ran in import and export are reflected in the observation that Ran-GTP, predicted to be at high concentration in the nucleus, stabilizes export receptor–cargo complexes but dissociates import receptor–cargo complexes (see Rexach and Blobel, 1995; Fornerod *et al.*, 1997a; Cole and Hammel, 1998; Kutay *et al.*, 1998, and references therein). Transport is regulated further by additional accessory factors, such as the protein p10/NTF2 (Moore and Blobel, 1994; Paschal and Gerace, 1995; Corbett and Silver, 1996; Clarkson *et al.*, 1997; Feldherr *et al.*, 1998).

With much of the groundwork laid in terms of identifying receptor–cargo transport complexes, one can now turn to the question of how this traffic moves through the pore. Fundamental to our understanding of nuclear pore function is the question of whether the importin β-related superfamily members have diverged in their capacity to interact with the transport machinery of the pore. Transport receptors have been observed to interact with many nucleoporins when tested in vitro (Radu *et al.*, 1995b; Aitchison *et al.*, 1996; Bonifaci *et al.*, 1997; Pemberton *et al.*, 1997; Percipalle *et al.*, 1997; Rosenblum *et al.*, 1997; Rout *et al.*, 1997). Although this provides information on a range of potential interactions, the issue of which interactions take place in vivo is at the crux of understanding how transport of different substrates takes place mechanistically.

In vivo, interactions are constrained by several factors including accessibility, as well as relative affinities when multiple binding choices are present. One way that in vivo interactions have been studied has been by examining competition between transport receptors for nuclear rim binding in permeabilized cells (Görlich *et al.*, 1997; Kutay *et al.*, 1997). Such studies have revealed that importin β and several other import receptors potentially bind overlapping sites at some point during transport. In vivo methods that focus on steady-state transport receptor–nucleoporin interactions have perhaps been more revealing of unique high-affinity interactions between given receptors and nucleoporins. For example, exportin 1 has been found to be a major binding partner of nucleoporin Nup214 in vivo (Fornerod *et al.*, 1997b), whereas importin β is found to interact stably with Nup358, Nup153, and Tpr but not other tested nucleoporins (Shah *et al.*, 1998). In vivo interactions have also been assessed genetically and biochemically in yeast (Belanger *et al.*, 1994; Iovine *et al.*, 1995; Simos *et al.*, 1996; Bailer *et al.*, 1998; Senger *et al.*, 1998). Extrapolation of

interactions found in yeast to the vertebrate pore is often difficult because of the divergence in pore proteins, but the results clearly lend further support to the conclusion that, in vivo, transport receptors interact preferentially with subsets of nucleoporins. Importantly, this indicates that different cargo–receptor complexes are likely to have distinct modes of transport through the pore. However, with the limited amount of information at hand, it is not yet clear how many distinct routes there are. At one extreme, there could be simply an import route and an export route. At the other extreme, there could be a distinct route through the pore for each class of cargo–receptor complex.

In a complementary approach, individual components of the pore can be assessed for their involvement in different types of transport. These experiments serve both to distinguish transport routes as well as to functionally define the pore transport machinery in vivo. The vertebrate nuclear pore presents a formidable experimental challenge because of its large size (120 million daltons) and complexity. Electron microscopy has revealed several striking features of the pore (see Goldberg and Allen, 1995; Yang *et al.*, 1998, and references therein). In addition to a central transporter region held within a large ring of spokes, asymmetric structures extend into the nucleus and cytoplasm. Filaments are found tethered on the cytoplasmic face of the pore, whereas other fibers extend to form a basket-like structure on the nucleoplasmic side of the pore.

Several individual components of the vertebrate pore have been molecularly identified, and their locations have been mapped to these structural landmarks. Briefly, Nup358, Nup214, and Nup84 (Nup88) are localized to the cytoplasmic filaments (Kraemer *et al.*, 1994; Wu *et al.*, 1995; Yokoyama *et al.*, 1995; Bastos *et al.*, 1997; Cordes *et al.*, 1997), whereas Nup62 and its associated binding partners (Nup58, Nup54, and Nup45) are located centrally (Grote *et al.*, 1995; Guan *et al.*, 1995). Nup153 has been localized by immunoelectron microscopy to the distal ring of the nuclear basket and intranuclear filaments (Cordes *et al.*, 1993; Sukegawa and Blobel, 1993; Panté *et al.*, 1994). Nup93, Nup98, and Nup205 are found on the nuclear face of the pore (Powers *et al.*, 1995; Radu *et al.*, 1995b; Grandi *et al.*, 1997), whereas the Tpr protein is found on filaments that extend from the pore basket deeper into the nucleus (Cordes *et al.*, 1997; Zimowska *et al.*, 1997). Although many pore proteins have yet to be identified, valuable clues to pore function can be gathered from studying the nucleoporins identified to date.

Results of studies focusing on Nup153 have been particularly intriguing. Bastos *et al.* (1996) found that overexpression of either full-length or certain truncations of Nup153 results in nuclear poly(A)+ RNA accumulation, suggesting that Nup153 plays a role in mRNA export. Studies in our laboratory, however, have revealed that within assembled pores, Nup153 is a major binding partner for importin β, indicating a role for Nup153 in import. Indeed, addition of a fragment of Nup153 to a permeabilized cell assay completely blocks importin β-mediated import (Shah and Forbes, 1998). To gain insight into the role of Nup153 in nuclear export, we have in this study probed numerous export, as well as import, pathways in *Xenopus* oocytes for defects following nuclear injection of antibodies specific for Nup153. A dramatic effect on multiple types of export is observed, while nuclear import and recycling take place normally. These results define a critical role for Nup153 in specific nuclear export pathways, a role that is prevented by antibody binding. We further find that Nup153 associates, either directly or indirectly, with certain homoribopolymers in vitro, providing a clue to the role Nup153 plays in export and distinguishing Nup153 mechanistically from other nucleoporins involved in RNA export.

MATERIALS AND METHODS

Plasmid Constructs and Oligos

Plasmids used as templates for the in vitro transcription of radiolabeled export substrates and the restriction enzyme used to linearize the plasmid were as follows: pBSAd (adenoviral major late premRNA [see Powers *et al.*, 1997]) and *Eco*RI; pT7–5S (provided by J. Gottesfeld [see You *et al.*, 1991]) and *Dra*I; pAd48 (Rev response element [RRE]-containing adenoviral major late premRNA, provided by U. Fischer [see Fischer *et al.*, 1994]) and *Bam*HI; and pBS-DHFR and *Xba*I. The latter plasmid was constructed by placing the cDNA sequence of DHFR (obtained from a construct provided by T. Hope, Salk Institute) into the *Bam*HI site of Bluescript. PCR was used to generate DNA templates to be used to direct in vitro transcription of tRNA, U1, and U6 snRNA transport substrates. The 5′ oligos used for the PCR contained either a T7 or SP6 phage polymerase promoter, and the genes were amplified from their respective plasmid DNAs. The oligonucleotides and corresponding plasmids for generating U6 and U1 snRNA templates were as described (Terns *et al.*, 1992, 1993). The in vitro transcription template for human $tRNA^{met}$ was generated with the following oligonucleotides: 5′-oligo (5′-TAATACGACTCACTATAGGGAGCAGAGTGGCGC-AGC) and 3′-oligo (5′-TAGCAGAGGATGGTT). M. Zasloff kindly provided a plasmid containing this tRNA gene (phH2D [Zasloff, 1983]).

A plasmid containing a T7-driven gene for pyruvate kinase (PK) fused at its C-terminal end to a bipartite NLS derived from hnRNPK (Michael *et al.*, 1995) was used to produce radiolabeled protein. The NLS was deleted from this construct to generate a DNA template that produced control PK protein (kindly provided by S. Vasu, D. J. Forbes laboratory). The *Xenopus* hnRNPA1 gene (a kind gift from M. Michael) was cloned into the CS2MT+ vector (Turner and Weintraub, 1994) between the *Nco*I and *Xho*I sites to generate an SP6-driven template for hnRNPA1 production. Finally, luciferase protein was made using a T7-luciferase control template (Promega, Madison, WI).

Bacterial expression constructs for Nup153 fragments are described in Shah *et al.* (1998) in which they are referred to as construct 3 (amino acids 53–334 of the partial *Xenopus* Nup153 sequence) and construct 1 (amino acids 334–828). The Gst–Rev expression construct used to produce Rev was the kind gift of M. Malim (Meyer *et al.*, 1996); the importin β expression construct was generously provided by D. Görlich (Kutay *et al.*, 1997).

Generation and Affinity Purification of Anti-Nup153 Antibodies

Purified recombinant Nup153 fragments corresponding to amino acids 53–334 and 334–828 were used to immunize rabbits for Ab1(380) and Ab2(363), respectively (Shah *et al.*, 1998). Neither antibody recognizes other FG repeat-containing nucleoporins, such as Nup358 or Nup62, on immunoblots. To affinity purify and concentrate antibodies for the work described here, the respective antigen was coupled to CnBr-activated Sepharose (Amersham, Arlington Heights, IL). Serum was diluted with an equal volume of 1 M NaCl, 0.4% Triton X-100, and 50 mM Tris (pH 8) and passed over the appropriate affinity matrix. The columns were washed with five volumes of 0.5 M NaCl, 0.2% Triton X-100, and 50 mM Tris (pH 8), followed by five volumes of PBS. Antibodies were then eluted with up to 20 volumes of 100 mM glycine (pH 2.5). The eluate was rapidly neutralized with a 1:10 volume of 1 M Tris (pH 8) and concentrated in a spin concentrator (10,000 molecular weight [MW] cutoff; Millipore, Bedford, MA). The buffer was then exchanged by diluting and reconcentrating three times with 10 volumes of PBS. Control preimmune antibodies were purified following standard procedures using Protein A beads (Harlow and Lane, 1988).

Production and Purification of Recombinant Proteins

Bacteria (BL21/DE3) containing the Nup153 expression constructs were induced with 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) in LB media for 3 h at 37°C. After standard procedures were followed for sonication and clarification, recombinant protein was purified on Ni-NTA-agarose according to the manufacturer's protocol (Novagen, Madison, WI). After dialysis into PBS, sucrose was added to 0.25 M before freezing. Recombinant importin β was expressed and purified as above, using the M15 strain of *Escherichia coli* (Qiagen, Santa Clara, CA), with growth and induction performed at room temperature. GST–Rev was produced according to Kellogg *et al.* (1995) in bacteria grown in NZ media at room temperature. Protein expression was induced with 0.1 mM isopropyl-β-D-thiogalactopyranoside for 3–4 h. Bacterial pellets were washed with cold PBS and sonicated after resuspension and a freeze/thaw cycle in cold PBS plus 0.5 mM PMSF and 5 μg/ml aprotinin and leupeptin. The resulting suspension was clarified by centrifugation and incubated with glutathione–sepharose (Amersham) pre-equilibrated in PBS. After 60 min at 4°C, the beads were washed extensively with PBS. Fusion protein was then eluted with 20 mM glutathione in Tris (pH 8), 120 mM NaCl, and 10 mM DTT, followed by buffer exchange to PBS and concentration in a spin concentrator (5000 MW cutoff; Millipore). Glycerol was added to 5% before freezing.

Preparation of Radiolabeled Transport Substrates

Radiolabeled RNA export substrates were transcribed in vitro following standard procedures (Melton *et al.*, 1984). To radiolabel import substrates and control proteins, plasmids containing SP6- or T7-driven genes for hnRNPA1, PK-NLS, PK, and luciferase were transcribed and translated using TnT rabbit reticulocyte lysate (Promega) in the presence of [^{35}S]-methionine (Dupont–New England Nuclear, Boston, MA). Unincorporated methionine was removed by twice diluting the sample in 500 μl of PBS and reconcentrating in a spin concentrator (5000 MW cutoff; Millipore). Glycerol was added to a 5% final concentration before freezing.

Injection and Harvest of Xenopus *Oocytes*

A small piece of ovary was surgically removed from a *Xenopus* frog and placed into modified Barth's solution. Stage V and VI oocytes were then selected and stored for up to 2 d at 18°C in modified Barth's solution. Immediately before injection, the oocytes were placed in a microwell miniplate (Nalge Nunc International, Naperville, IL), with animal poles oriented upward, and centrifuged at 1200 $\times$ *g* for 10 min at ~22°C. Microinjections were then performed, ~10 nl if nuclear and 30–50 nl if cytoplasmic. Blue dextran 2000 (18 mg/ml; Amersham) or, in some instances, rabbit reticulocyte lysate (20%; Promega) was included with the injected material to visually distinguish successful nuclear injections after dissection. Incubations were performed at 18°C for the duration indicated in each experiment. The oocytes were then manually dissected under mineral oil into nuclei and cytoplasm (Lund and Paine, 1990), and each fraction was transferred into the appropriate buffer with a microcapillary pipette. For isolation of RNA, the buffer used was 100 mM Tris (pH 7.5), 300 mM NaCl, 10 mM EDTA, and 2% SDS. For isolation of protein, the buffer used was 65 mM Tris (pH 7.5), 10 mM EGTA plus 1 mM DTT, and 1 mM PMSF. Generally, material from two to three oocytes was combined, and wherever possible, samples were collected and analyzed in duplicate.

Preparation and Analysis of Injected RNA

After dissection of oocytes, the RNA was prepared for gel analysis by protease K digestion for 60–90 min at 37°C (1 mg/ml; Worthington Biochemical, Lakewood, NJ), followed by extraction with phenol/chloroform and precipitation with ethanol. Glycogen (30 μg; Boehringer Mannheim, Indianapolis, IN) was added to nuclear samples as a carrier. The RNA pellets were resuspended in gel-loading buffer (80% formamide, 1$\times$ TBE buffer) and heated to 65–70°C for ~10 min. The material equivalent to the amount found in one oocyte nucleus or cytoplasm was loaded in each lane of a denaturing acrylamide gel (6% acrylamide, 6 M urea, TBE). After electrophoresis, the gel was fixed and dried. The results were analyzed by autoradiography and quantitative phosphorimaging (Molecular Dynamics, Sunnyvale, CA).

Preparation and Analysis of Injected Proteins

To examine protein localization, we homogenized oocyte nuclei and cytoplasm samples either by repetitive pipetting or with a disposable mini pellet pestle (Fisher Scientific, Pittsburg, PA). After centrifugation in a horizontal microfuge rotor (5 min, 4°C, 7500 $\times$ *g*), 75% of the material was transferred to a new tube, avoiding the yolk protein–containing pellet. Total protein was precipitated by the addition of three to four volumes of acetone, with 40 μg of soybean trypsin inhibitor added to nuclear fractions as a carrier. After centrifugation, the resulting pellets were thoroughly dried at ~65°C, and then SDS–Laemmli sample buffer was added to each tube. Protein was resuspended by warming to 37°C for 60 min and gently vortexing. After a 10 min incubation at 99°C, the samples were electrophoresed on SDS–Laemmli gels. Material equivalent to 88% of one nucleus and 22% of one cytoplasm was loaded on the gel, unless otherwise noted. This corresponds to 44 nl of nuclear material and 110 nl of cytoplasmic material, based on a 50- and 500-nl volume, respectively. When ^{35}S-labeled proteins were being monitored, gels were fixed, amplified (Amplify; Amersham), and dried, and the results were visualized by autoradiography. Otherwise, the proteins were electrophoretically transferred to polyvinylidene fluoride membrane (PVDF; Millipore) for Western blot analysis. The filter was placed in blocking solution (5% dried nonfat milk, 2% BSA, 0.1% Tween in PBS for 60 min at room temperature for anti-GST immunoblotting; and in 1% casein, 0.1% Tween, PBS overnight at 4°C for anti-6XHis immunoblotting). Incubation with primary antibody was performed overnight (4°C) in 0.1% Tween and PBS (anti-GST) or in 1% casein, 0.1% Tween, and PBS for 2 h at room temperature (anti-6XHis; Tetra mAb; Qiagen). The primary antibody was detected using an HRP-conjugated secondary antibody (Jackson ImmunoResearch Laboratories, West Grove, PA) and chemiluminescent substrate (Renaissance ECL; Dupont–New England Nuclear).

Identification of Polyribonucleotide-Binding Nucleoporins

High-speed extract prepared from *Xenopus* eggs (see Smythe and Newport, 1991) was diluted to 2 mg/ml in 25 mM HEPES, 100 mM KCl (or more, where indicated), and aprotinin/leupeptin (5 μg/ml). Nonidet P-40 (NP-40) was also included as indicated. Beads, either uncoupled or coupled to each of the homoribopolymers [poly(G), poly(C), and poly(A) (Sigma, St. Louis, MO) and poly(U) (Pharmacia)] were equilibrated (30 min) in the same buffer and then incubated with the diluted *Xenopus* extract for 45–60 min at 4°C. To reduce nonspecific binding, BSA (10 mg/ml) was included in the pre-equilibration and binding incubations where noted. The RNA resins and associated proteins were separated from unbound proteins by centrifugation (14,000 × *g*, 30 sec, 4°C), and the beads were washed, first with buffer containing heparin (2 mg/ml, 10 min, 4°C) and then with buffer alone (five rapid washes, with KCl omitted from the last wash). Proteins that remained associated with the resins were eluted in SDS sample buffer and analyzed by SDS–gel electrophoresis, followed by Western blotting. Immunoblotting was performed as described above, except 5% nonfat dried milk with 0.2% Tween in PBS was used as the blocking solution and either mAb414 (Babco, Berkeley, CA) or affinity-purified anti-Nup98 antibodies were used as primary antibodies.

RESULTS

Nup153 Plays a Key Role in the Export of Multiple RNA Cargo

To characterize the role of Nup153 in nuclear transport, we attempted to interfere with Nup153 activity in vivo by the injection of specific antibodies. Affinity-purified anti-Nup153 antibodies, control buffer, or preimmune IgG were injected into the nuclei of *Xenopus* oocytes (Figure 1A). One hour later, radioactively labeled RNA export substrates were microinjected into the same nuclei. After a further 2 h incubation, the oocytes were dissected into nuclei and cytoplasm, and RNA was prepared from each fraction and analyzed by gel electrophoresis. In a first experiment, radiolabeled pre-mRNA, U1 snRNA, 5S RNA, and U6 snRNA were used (Figure 1B). U6 RNA typically is retained in the nucleus and serves as a control for accurate nuclear injection (Hamm and Mattaj, 1989). Here, U6 was found to be exclusively nuclear (Figure 1B, lanes 1, 3, and 5). The injected pre-mRNA transcript is normally processed in vivo into mature mRNA and an intron lariat. The intron lariat was not transported to the cytoplasm in the buffer-injected control oocytes (Figure 1B, lanes 1 and 2); however, a substantial amount of the spliced mRNA was efficiently exported by the 2 h time point (Figure 1B, lane 2, mRNA). Similarly, significant amounts of U1 and 5S RNA were exported to the cytoplasm in the buffer-injected control ooctyes (Figure 1B, lane 2). This overall pattern of export was also seen when IgG, isolated from preimmune serum, was injected into nuclei (Figure 1B, lanes 3 and 4). In contrast, when antibodies directed to Nup153 were injected into nuclei (Figure 1B, lanes 5 and 6), the export of mRNA, U1 snRNA, and 5S RNA each was strikingly inhibited.

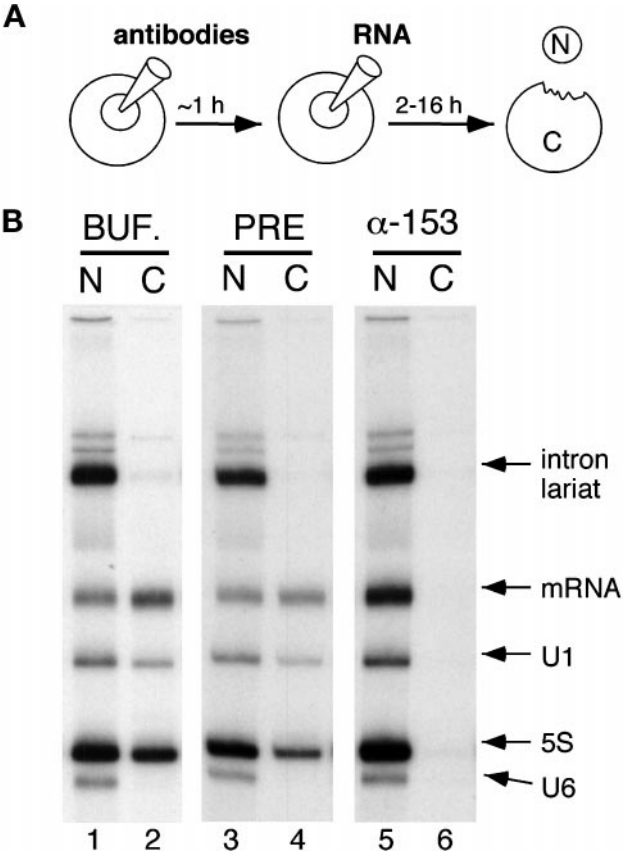

Figure 1. Antibodies to Nup153 prevent the export of multiple classes of RNA. (A) The oocyte injection protocol is shown. First, antibodies were injected into the nucleus. After a 60 min incubation, a second nuclear injection was performed, delivering radioactively labeled RNA. The oocytes were incubated for periods of varying duration and then manually dissected. Nuclear (N) and cytoplasmic (C) RNA was prepared and analyzed by gel electrophoresis. (B) After initial injections either with buffer (BUF., lanes 1 and 2), preimmune IgG (PRE, lanes 3 and 4), or affinity-purified antibodies to Nup153 (α-153, lanes 5 and 6), oocytes were next injected with a mixture of RNA (pre-mRNA, U1 snRNA, 5S rRNA, and U6 snRNA). Two hours later, nuclear and cytoplasmic RNA was harvested. The pre-mRNA has been processed into mature mRNA and a lariat intron. The intron, as well as U6 snRNA, is not normally exported; their nuclear localization here confirms the accuracy of the injection and dissection. Nuclear export of mRNA, U1 snRNA, and 5S RNA is substantially underway at this 2 h time point (lanes 2 and 4) but is fully prevented in the presence of anti-Nup153 antibodies (lane 6).

The effect of anti-Nup153 antibodies on tRNA export was next addressed. Although once again the export of both mRNA and U1 snRNA was disrupted by anti-Nup153 antibodies, tRNA export took place at its normally rapid rate in the presence of the Nup153 antibodies (Figure 2A, compare lanes 3 and 4 with lanes 1 and 2). Because anti-Nup153 antibodies clearly have an inhibitory effect on the export of mRNA, 5S RNA, and U1 snRNA, we next wanted to ascertain that this inhibitory phenotype was indeed attributable to the immunoreactivity of the antibodies and not

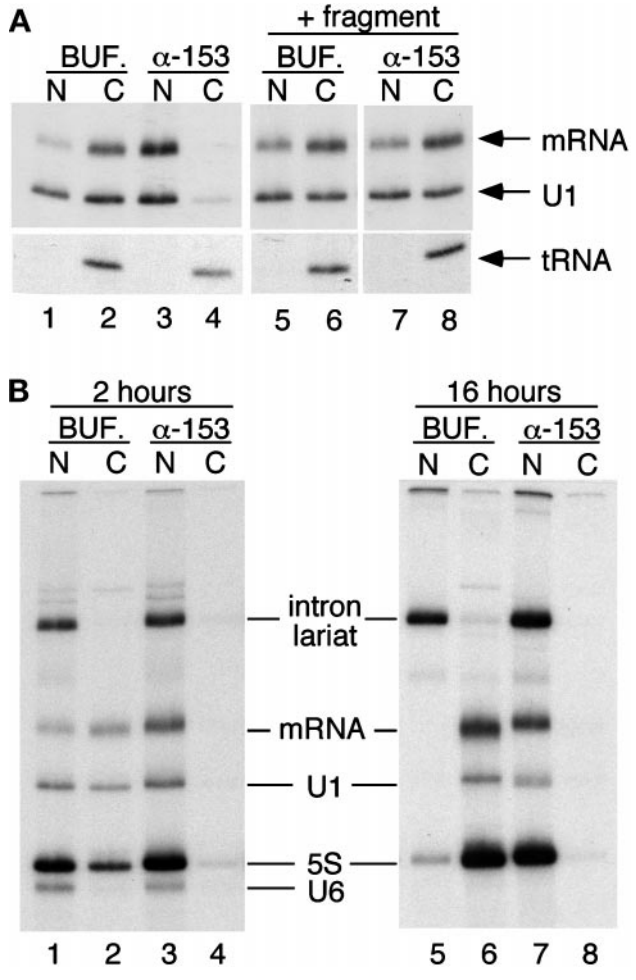

Figure 2. Antibodies to Nup153 have a long-lasting and specific inhibitory effect on mRNA export but fail to block tRNA export. (A) An injection experiment was performed as described in Figure 1, except that, in some cases, a recombinant fragment of Nup153 was coinjected with the first injection of buffer or antibodies (lanes 5–8). The mix of RNAs in this experiment included tRNA in addition to pre-mRNA and U1 snRNA. Antibodies to Nup153 did not prevent tRNA export (compare lanes 2 and 4), whereas, as before, export of mRNA and U1 snRNA was blocked by these antibodies. When the Nup153 fragment was coinjected with anti-Nup153 antibodies (lanes 7 and 8), the effect of the antibody was neutralized (lane 8). (B) After injection of either buffer or antibodies, oocytes were injected with a mixture of pre-mRNA, U1 snRNA, 5S RNA, and U6 snRNA and then harvested after either 2 h (lanes 1–4) or 16 h (lanes 5–8) of incubation at 18°C. U6 snRNA is relatively less stable and is no longer detected at the later time point. Export of mRNA, U1 snRNA, and 5S RNA was inhibited at both early and late time points in oocytes preinjected with antibodies to Nup153.

attributable to any nonspecific inhibitor associated with the affinity-purified antibodies. Toward this end, we examined whether coinjection of the recombinant Nup153 protein fragment (amino acids [aa] 53–334) to which the antisera was raised could reverse the effect of antibody injection. When recombinant protein (10 ng) was mixed with anti-Nup153 antibodies before injection, mRNA and snRNA export now took place normally (Figure 2A, lane 8), demonstrating the specificity of antibody inhibition. Another important observation is that in this experiment the Nup153 fragment alone (Figure 2A, lane 6) had no dominant-negative effect on RNA export.

A question that is raised by these results is whether the Nup153 antibodies actually *block* RNA export or whether they simply slow the kinetics of export. To address this question, we harvested RNA from oocytes incubated for 16 h after the injection of RNA. Even after this long incubation period, mRNA, 5S RNA, and U1 snRNA export remained quantitatively inhibited by the anti-Nup153 antibodies (Figure 2B, compare lanes 4 and 8). This sustained block to export indicates that the antibodies prevent an essential step in the export process.

Nup153 Is Critical to NES-directed Export

We next wished to investigate directly the role of Nup153 in NES-directed protein export from the nucleus. In terms of the export signal and corresponding soluble receptor, NES-directed protein export is a relatively well-characterized export pathway, with the prototypical export cargo being the HIV Rev protein. When injected into the nucleus of a *Xenopus* oocyte, GST–Rev is normally exported to the cytoplasm (Figure 3A, lane 2) (Fornerod *et al.*, 1997a). Injection of anti-Nup153 antibodies before the injection of GST–Rev, however, resulted in a significant block to Rev export (Figure 3A, lane 4). Although the extent of this inhibition varied somewhat, the inhibition was consistently observed. Thus, Nup153 must normally contribute to NES-directed protein export.

The HIV Rev protein is critical to the viral life cycle because it facilitates the export of unspliced and partially spliced viral transcripts. This transport step is needed to express the full complement of viral proteins, as well as to propagate the viral genome. Unspliced and partially spliced viral transcripts contain an RNA recognition sequence known as the RRE. Rev proteins bind to this sequence, and the resulting protein–RNA complexes are then exported, escaping nuclear retention typical for endogenous transcripts that are only partially processed (for review, see Hope, 1997). In terms of the mechanism of transport through the pore, it is possible that there are differences between the export of Rev alone, as opposed to a Rev–RNA complex. Certainly these two export cargos are significantly different in terms of size, as well as the number of NESs per cargo complex. We therefore used the Nup153 antibodies to address whether Nup153 also contributes to the Rev-mediated RNA export pathway.

To assess an effect on Rev-mediated RNA export, a radiolabeled adenoviral premRNA engineered to con-

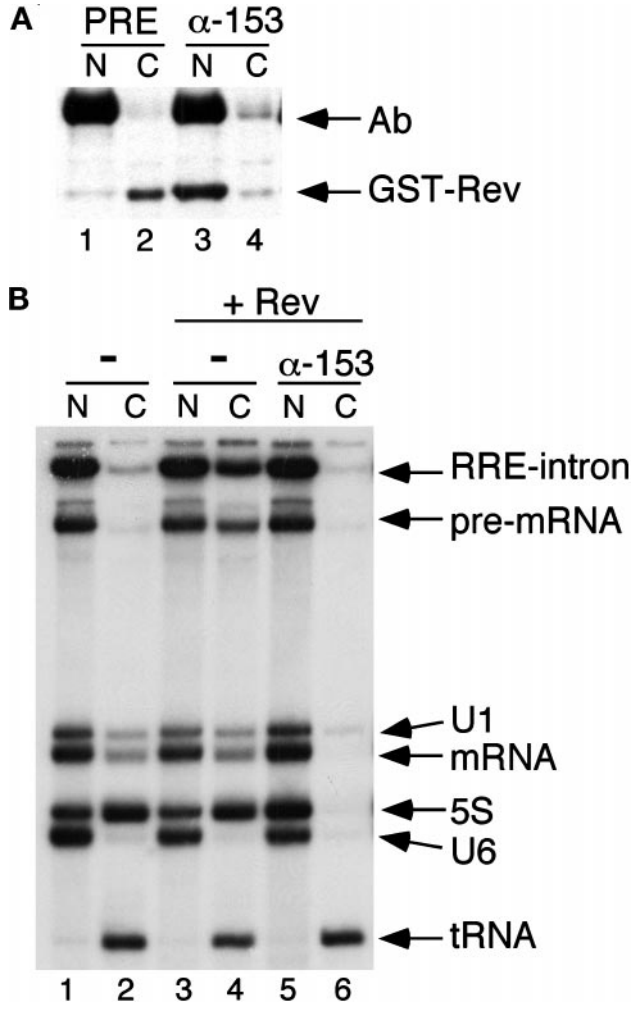

Figure 3. Nup153 is required for Rev protein export and Rev-mediated RNA export. (A) After injection with either preimmune IgG (lanes 1 and 2) or antibodies to Nup153 (lanes 3 and 4), a recombinant form of the HIV protein Rev (GST–Rev) was injected into the oocyte nuclei. After 2 h, protein was harvested from dissected oocytes and analyzed by Western blot. Rev protein export was inhibited by anti-Nup153 antibodies (compare lanes 2 and 4). The preimmune and affinity-purified antibodies injected into the oocyte nuclei are detected by the secondary antibody (goat anti-rabbit IgG) used in the Western blot (lanes 1 and 3, Ab). (B) Control and antibody-injected oocytes were injected with a mixture of pre-mRNA, U1 snRNA, 5S RNA, U6 snRNA, and tRNA. The pre-mRNA used here contains a Rev response element (RRE) in the intron. Where indicated, GST–Rev was also included in the RNA mixture (lanes 3–6). In the presence of GST–Rev, the RRE-containing lariat intron as well as the pre-mRNA was exported (compare lanes 2 and 4). However, antibodies against Nup153 block Rev-mediated RRE export (lane 6).

tain an RRE in the intron (Fischer *et al.*, 1994) was injected into oocyte nuclei in the presence or absence of GST–Rev (Figure 3B). As had been demonstrated previously, Rev-mediated export was faithfully recapitulated in the oocytes; the RRE-containing intron, as well as the pre-mRNA, was exported to the cytoplasm in the presence of Rev (Figure 3B, lane 4) but not in its absence (Figure 3B, lane 2). In contrast, coinjection of a mutant form of Rev (M10) that contains an inactive NES (Malim *et al.*, 1989) did not promote export of RRE-containing RNA (Fischer *et al.*, 1994; our unpublished results). Although Rev-mediated export shares a limiting factor(s) with other export pathways (Fischer *et al.*, 1995; Pasquinelli *et al.*, 1997b), at the low levels of Rev used here Rev had no competitive effect on U1 snRNA, spliced mRNA, 5S RNA, or tRNA export (Figure 3B, compare lanes 2 and 4) (see also Fischer *et al.*, 1994, 1995). When Rev-mediated RNA export was examined after injection of antibodies to Nup153, Rev–RNA export was found to be markedly inhibited (Figure 3B, lane 6, RRE-intron RNA and premRNA), indicating that Nup153 plays an important role in the export of both Rev itself and a Rev–RRE–RNA complex.

Nuclear Import Occurs Normally in the Presence of Anti-Nup153 Antibodies

Having established that anti-Nup153 antibodies specifically inhibit multiple export pathways, we wished to assess the effect of these antibodies on nuclear import. For this, we again injected anti-Nup153 antibodies into the nucleus and, after a 60 min incubation, injected radioactively labeled import substrate into the cytoplasm (Figure 4A). To test import directed by a classical NLS, we used a chimeric protein containing pyruvate kinase (PK) fused to a basic bipartite NLS derived from hnRNPK (Matunis *et al.*, 1992; Michael *et al.*, 1995). Although wild-type PK remained in the cytoplasm when examined 4 h after injection (Figure 4B, lanes 1 and 2), the PK–NLS protein was found in both compartments at 4 h (Figure 4B, lanes 3 and 4). Notably, this NLS-directed import took place equally well when the oocytes had been injected with antibodies against Nup153 (Figure 4B, lanes 5 and 6).

Importin α and β mediate the import of NLS-containing proteins, such as the test substrate above. However, there are other distinct import pathways mediated by different import receptors. To examine a second, independent import pathway, we injected hnRNPA1. hnRNPA1 has a nonclassical nuclear import signal termed M9 that interacts with the import receptor transportin (Pollard *et al.*, 1996). After cytoplasmic injection of hnRNPA1, a significant amount was imported into nuclei by 4 h (Figure 4C, lanes 1 and 2). Coinjected luciferase control protein remained primarily cytoplasmic (lane 2). When anti-Nup153 antibodies were injected, the nuclear import of hnRNPA1 took place with equal efficiency (Figure 4C, compare lanes 1 and 3). Thus, under conditions in which multiple export pathways are inhibited, both

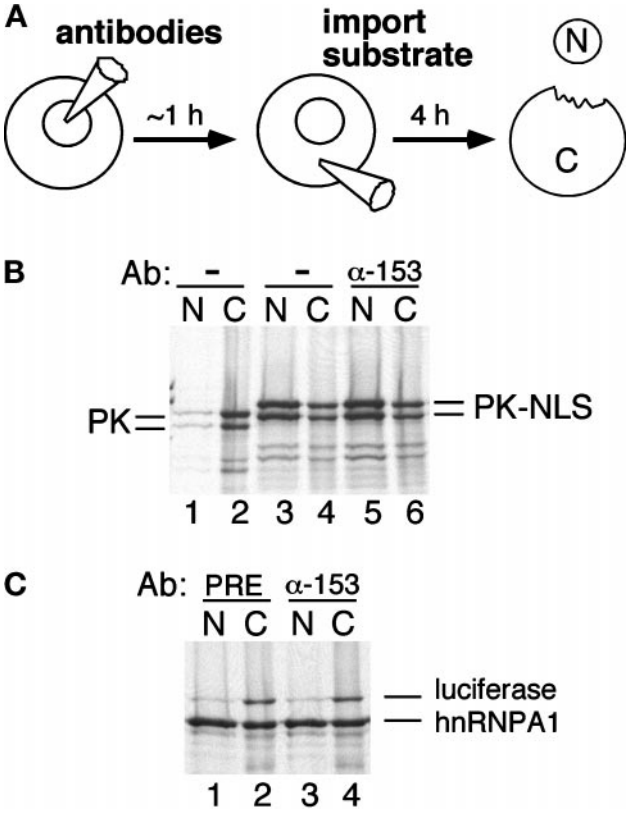

Figure 4. Nuclear import occurs in the presence of antibodies to Nup153. (A) The oocyte injection protocol is shown. Antibodies were injected into the nucleus, and after a 60 min incubation, radioactively labeled import substrates were injected into the cytoplasm. After 4 h of incubation, oocytes were manually dissected, and nuclear and cytoplasmic protein was prepared and analyzed by gel electrophoresis. (B) After cytoplasmic injection, pyruvate kinase (PK) remains primarily cytoplasmic (lanes 1 and 2). In contrast, a substantial amount of an NLS-containing form of PK migrates to the nucleus after cytoplasmic injection (lanes 3 and 4). This NLS-directed import continues in the presence of anti-Nup153 antibodies (lanes 5 and 6). (C) M9-directed import of hnRNPA1 also took place in the presence of antibodies to Nup153 (lanes 3 and 4 vs. lanes 1 and 2). Luciferase, coinjected into the cytoplasm with hnRNPA1, remained primarily cytoplasmic, serving as a control for the specificity of nuclear accumulation.

NLS- and M9-mediated transport into the nucleus occurs normally.

Importin β Recycles in the Presence of Anti-Nup153 Antibodies

After a round of import has taken place, soluble import receptors must be recycled back to the cytoplasm. To examine whether the recycling phase of import occurs when anti-Nup153 antibodies are present, we examined the export of importin β. Recombinant importin β, tagged with a 6XHis epitope, was injected into oocyte nuclei. Oocytes were incubated for 2 or 17 h and then dissected and examined by Western

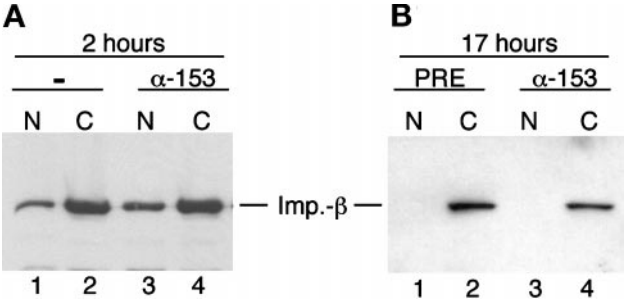

Figure 5. Importin β exports in the presence of anti-Nup153 antibodies. (A) After either no treatment or antibody injection, recombinant 6XHis-importin β (Imp.-β) was injected into the oocyte nuclei. The localization of His-tagged importin β was then examined at an early time point (2 h) when its export was still underway. The nucleocytoplasmic distribution of 6XHis-importin β was similar in oocytes that were either untreated (lanes 1 and 2) or injected with anti-Nup153 antibodies (lanes 3 and 4) before the injection of importin β. This indicates that the kinetics of importin β recycling are unaltered by the presence of antibodies to Nup153. (B) In an independent experiment, recombinant importin β was injected into nuclei after injection of either preimmune IgG or anti-Nup153 antibodies. Protein was harvested from nuclear and cytoplasmic fractions 17 h later. His-tagged importin β equilibrated to the cytoplasm even in the presence of Nup153-specific antibodies (lanes 2 and 4).

blotting. At a relatively early time point (2 h), a substantial portion of importin β injected into the nuclei of control oocytes had already been exported to the cytoplasm (Figure 5A, lane 2). Significantly, an equal proportion of importin β was found in the cytoplasm of oocytes injected with anti-Nup153 antibodies (Figure 5A, lane 4), indicating that importin β was exporting with identical kinetics in the absence or presence of the antibodies. At a later time point (17 h), importin β was found to have equilibrated predominantly to the cytoplasm, in oocytes injected either with control antibodies (Figure 5B, lane 2) or with Nup153-specific antibodies (Figure 5B, lane 4). Endogenous importin β also had a predominantly cytoplasmic localization in control, as well as in anti-Nup153–injected oocytes (our unpublished results). These results indicate that importin β is efficiently recycled to the cytoplasm in the presence of the Nup153 antibodies used here.

Antibodies Directed to Distinct Regions of Nup153 Have Differential Effects on Export

The data indicate that the export of very different substrates shares a requirement for access to Nup153, access that is blocked by the presence of anti-Nup153 antibodies. These different export complexes are likely to interact with Nup153—directly or indirectly—in their progression through the nuclear pore. This leads to the interesting question as to whether the antibodies disrupt an interaction shared by each of the export substrates or whether each substrate (or subsets thereof) has a unique interaction with Nup153. To

delineate further the requirements for Nup153 in export and to ask whether differences between the routes of different export substrates can be detected, we used a second set of anti-Nup153 antibodies raised to a different region of Nup153.

The antibodies in the previous experiments (now referred to as Ab1) were raised against a fragment of Nup153 that includes a unique N-terminal region and one zinc finger (Figure 6A, fragment 1; referred to as "Construct 3" in Shah *et al.* [1998]). A second antiserum (Ab2) was generated to fragment 2 (Figure 6A; "Construct 1" in Shah *et al.* [1998]) that includes the zinc fingers of Nup153 and part of the C-terminal FG domain located downstream of fragment 1. Affinity-purified Ab1 or Ab2 antibodies were injected into oocyte nuclei, followed by the injection of RNA export substrates U1 and tRNA and an additional class of RNA export substrate DHFR mRNA, made as an intronless transcript and therefore not processed in vivo. The export pathway of this latter type of mRNA has been shown to have some features distinct from that of spliced mRNA (Pasquinelli, *et al.*, 1997a,b; Saavedra *et al.*, 1997). In this experiment, DHFR RNA export proved to share a requirement with spliced mRNA for functional Nup153 in the pore; DHFR RNA export was strongly blocked by both Ab1 and Ab2 (Figure 6B, lanes 4 and 6; 90 and 96% inhibition, respectively, for DHFR export). In contrast, U1 RNA export was differentially affected by the anti-Nup153 antibodies. Although Ab1, as seen in previous experiments, was a potent inhibitor of U1 export (Figure 6, lane 4), Ab2 was not as effective at blocking U1 export (Figure 6B, lane 6; 85 and 40% inhibition in the presence of Ab1 and Ab2, respectively). tRNA export was robust in the presence of both Ab1 and Ab2 (Figure 6, lanes 4 and 6). The results of several experiments in which Ab1 and Ab2 were tested are shown in Figure 6C. Although spliced adenoviral mRNA and 5S RNA are comparably inhibited by both antibody types, U1 snRNA export was consistently less sensitive to Ab2 than to Ab1. This reveals that the export route of U1 has distinct requirements for Nup153 (see DISCUSSION).

Nup153 Associates with Poly(G) and Poly(U) Homoribopolymers

The central role of Nup153 in RNA export led us to ask whether Nup153 could associate, either directly or indirectly, with RNA. One method that has proven useful in detecting the affinity of a protein for RNA is to test its affinity for homoribopolymers. We therefore examined whether Nup153 could associate with poly(G), poly(C), poly(U), or poly(A) when the ribopolymer was coupled to a gel matrix. Extracts made from *Xenopus* eggs were incubated with each of the affinity matrices, as well as with control resin that had

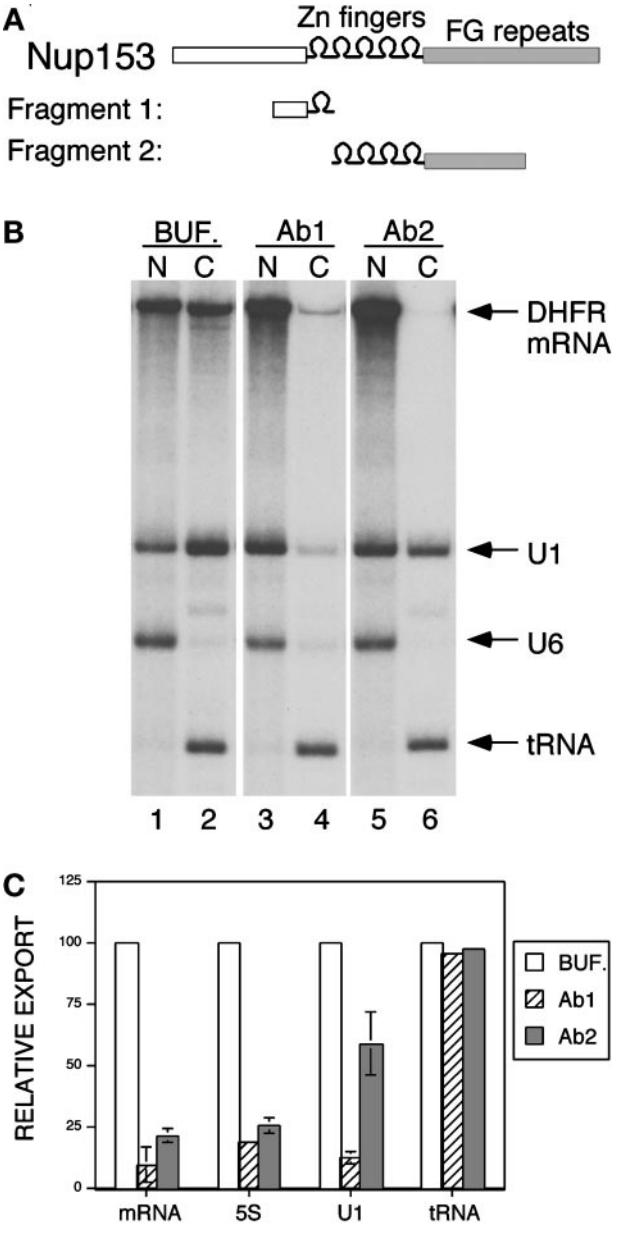

Figure 6. Antibodies to distinct regions of Nup153 affect export differentially. (A) The domain structure of *Xenopus* Nup153 is represented schematically, showing the unique N-terminal region, the five zinc fingers, and the FG-containing C-terminal region. Antibody 1 (Ab1) was raised against a fragment containing part of the N-terminal region and one zinc finger (amino acids 53–334). Antibody 2 (Ab2) was raised against the remaining four zinc fingers and part of the FG region (amino acids 334–828). (B) Oocytes were injected as described in Figure 1, first with buffer or antibodies and then with radiolabeled RNA. The RNA mixture used here contained DHFR mRNA (an intronless mRNA), U1 snRNA, U6 snRNA, and tRNA. RNA localization was analyzed after a 4 h incubation. DHFR mRNA export was inhibited equally by Ab1 and Ab2, whereas U1 RNA export is significantly less sensitive to Ab2 (compare lanes 2, 4, and 6). (C) The effects of Ab1 and Ab2 are depicted in this bar chart in which data from multiple experiments are summarized. Inhibitory profiles of antibodies to distinct regions of Nup153 are shown here for the adenoviral mRNA (spliced), U1 snRNA, 5S rRNA, and tRNA.

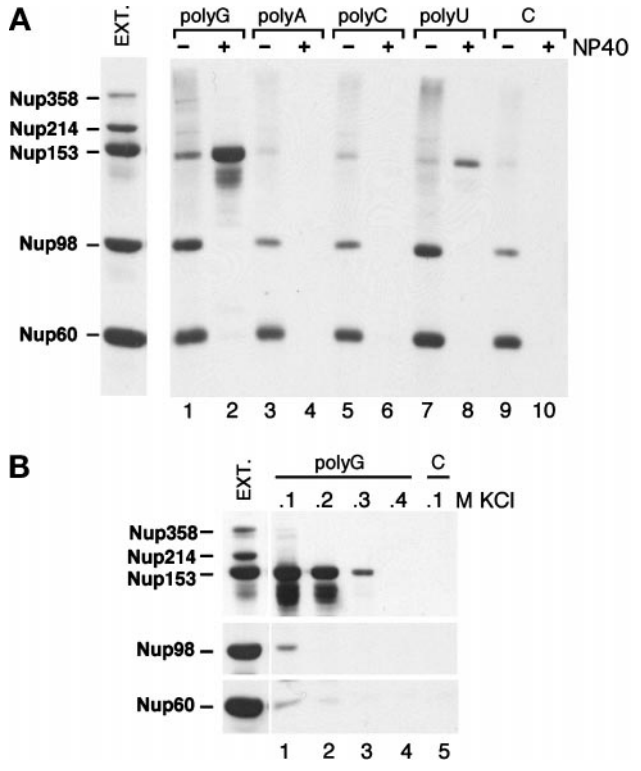

Figure 7. Nup153 interacts, directly or indirectly, with poly(G) and poly(U). (A) Affinity resins bearing the homoribopolymer indicated (lanes 1–8) or resin alone (lanes 9 and 10; control [C]) were incubated with *Xenopus* egg extract in the absence (lanes 1, 3, 5, 7, and 9) or presence (lanes 2, 4, 6, 8, and 10) of 0.5% NP-40. After the beads were washed (see MATERIALS AND METHODS), proteins that remained associated were eluted in SDS sample buffer and analyzed by Western blot. mAb414 and antibodies specific for Nup98 were used to detect a panel of nucleoporins. Nup358, Nup214, Nup153, Nup98, and Nup60 are all detected in egg extract (Ext. [left], an amount equivalent to 28% of the material included in each incubation is loaded). Some nonspecific binding of Nup98 and Nup60 is found in the absence of NP-40 when the nucleoporins recovered with the control beads are examined (lane 9). Levels over this background binding are clearly detected for Nup153 with poly(G) (lane 1), and this binding is greatly augmented by the presence of NP-40 (lane 2). Nup153 also associated with poly(U) in the presence of NP-40 (lane 8). (B) A binding experiment similar to that described above was performed with a matrix bearing the poly(G) ribopolymer and an identical control matrix lacking ribopolymer. Nucleoporin recovery was examined in the presence of increasing amounts of KCl and with NP-40 (0.2%) present. Nup153 remained associated with poly(G) when the KCl concentration was as high as 300 mM (lane 3). The left panel of egg extract represents 16% of the loaded material.

no nucleotide attached. Binding was performed in the presence or absence of the nonionic detergent NP-40. After a wash containing heparin to reduce nonspecific binding, the proteins associated with each affinity matrix were subjected to gel electrophoresis and analyzed by immunoblotting using antisera to nucleoporins. Although several nucleoporins associated with the different ribopolymer beads in the absence of NP-40 (Figure 7A, lanes 1, 3, 5, and 7), a substantial amount of the observed binding could be accounted for by the background levels of association with control beads (Figure 7A, lane 9). In most cases, this binding activity was lost when NP-40 was added to the extract before incubation with the resin (Figure 7A, lanes 2, 4, 6, and 8). However, Nup153 exhibited a high level of association with poly(G) in the presence of NP-40 (0.5%) (Figure 7A, lane 2) and a lesser but significant association with poly(U) under these conditions (Figure 7A, lane 8).

This binding of Nup153 to poly(G) and poly(U) far exceeded that of the other nucleoporins. To be specific, Nup358 and Nup214 did not appear to associate with any of the homoribopolymers even in the absence of NP-40. Nup60 had binding levels marginally over background to poly(G) and poly(U) (Figure 7A, compare control lane 9 with lanes 1 and 7). Nup98 appeared to have low levels of association with poly(G) and poly(U) compared with background, but for both Nup98 and Nup60, binding was disrupted by the presence of NP-40.

To look further at the stringency of the interaction between Nup153 and poly(G), the association was examined in buffer containing 0.2% NP-40 with increasing amounts of KCl. BSA (10 mg/ml) was included to reduce any nonspecific association with the beads. Nup153 was detected bound to poly(G) at concentrations of salt up to 300 mM (Figure 7B, lanes 1–3), whereas the low levels of Nup98 and Nup60 binding to poly(G) were highly sensitive to salt (Figure 7B, lanes 1 and 2). These experiments demonstrate a significant association of Nup153 with poly(G) and suggest that Nup153 may likewise associate with certain cellular RNAs, either by direct binding or indirectly by interacting with an adaptor or receptor protein involved in export. Such a protein is likely to be present in egg cytosol and would therefore be available if needed to bridge the binding of Nup153 to RNA.

DISCUSSION

To understand the process of nucleocytoplasmic trafficking, a major question that must be addressed is how traffic moves through the nuclear pore in a regulated, bidirectional manner. Little is known of the way in which individual proteins of the pore contribute to this complex process, yet this information is key to dissecting the mechanisms of transport. In this study, we have analyzed nuclear export and the role of the nucleoporin Nup153, the only nucleoporin known to be located on the distal ring of the pore basket, a location encountered very early in the export process. Our results indicate that Nup153 is a central component of the export machinery for multiple types of RNA and protein cargo. Specifically, antibodies to Nup153 block the export of U1 snRNA, 5S RNA,

spliced mRNA, and intronless mRNA. We find that Nup153 does not play the same critical role in the export of tRNA. Thus, Nup153 plays a key role in specific paths of RNA export. Equally importantly, antibodies against Nup153 were used to examine Rev protein export, as well as Rev-mediated RNA export. In both cases, export was inhibited, demonstrating that Nup153 plays a role in NES protein export and NES-mediated RNA export. These results indicate that although there may be mechanistic differences between NES *protein* export and NES-mediated *RNA* export, Nup153 is critical to the export of both types of cargo.

To assess further whether other transport pathways through the pore are disrupted by antibodies bound to Nup153, we examined two distinct routes of nuclear import. In this case, both NLS- and M9-mediated import pathways were found to be unperturbed by the anti-Nup153 antibodies used here. We also examined the recycling phase of nuclear import by analyzing the export of importin β to the cytoplasm. This factor was found to export normally in the presence of our Nup153-specific antibodies. Consistent with this observation, endogenous importin β remained in its primarily cytoplasmic location in the presence of the anti-Nup153 antibodies (our unpublished results); the antibodies would have caused relocalization of importin β to nuclei if recycling had been inhibited. Our experiments clearly demonstrate that, although antibodies disrupt a crucial function of Nup153 with respect to a number of export pathways, several modes of traffic through the pore continue to take place normally. This underscores the specificity of the export block we see in the presence of anti-Nup153 antibodies.

Nup153 has been found to be a major in vivo binding partner of importin β (Shah *et al.*, 1998). A fragment of Nup153 containing the first half of the FG region of Nup153 (equivalent to hNup153 aa 976-1198), when added in excess, completely blocks importin β-mediated import in permeabilized cells. Moreover, a more N-terminal fragment of Nup153 (equivalent to hNup153 aa 431–723), when added in excess, blocks transportin-mediated import (Shah and Forbes, 1998). Both Nup153 fragments bind the respective receptors in vitro. As such, Nup153 appears to play a role in the last steps of import. The experiments here do not address the in vivo role of the Nup153–import receptor interactions, because the Nup153 antibodies used here do not interfere with the Nup153–importin β or Nup153–transportin interactions. This is clear from the finding that the Nup153 antibodies coimmunoprecipitate importin β and transportin (Shah and Forbes, 1998; Shah *et al.*, 1998), indicating that the antibodies do not displace the import receptors from Nup153. Moreover, when the levels of Nup153 associated with exogenous importin β in *Xenopus* extract were compared in the absence or presence of our Nup153 antibodies, no significant differences were observed (our unpublished results). This was the case with both Ab1 and Ab2, providing unequivocal evidence that the antibodies do not interfere with the Nup153–importin β interaction in egg extract. This is likely attributable to the fact that there are multiple importin β-binding sites in the FG-repeat region of Nup153 (Shah *et al.*, 1998). Ab2 was raised to the proximal half of the FG region and is likely to bind exclusively to it, leaving the downstream importin β-binding site accessible and functional for roles in β-mediated import and/or β recycling. Thus, the results illustrate that not only do the antibodies affect Nup153 specifically, they target only certain interactions involving this nucleoporin, those involved in the *export* of a specific subset of cargo.

It is interesting that this subset of export pathways is blocked by two different anti-Nup153 antibodies, one raised to a unique proximal region of Nup153 plus the first of the Zn fingers and a second antibody raised to a more distal fragment consisting of a nonoverlapping portion of the Zn finger domain and the first half of the FG region (Figure 6). Ab1 and Ab2 both inhibit the export of multiple RNAs. However, one striking difference between the antibodies is the relative refractory nature of the U1 export pathway to Ab2 inhibition. Specifically, spliced adenoviral mRNA, intronless DHFR mRNA, and 5S RNA export were all inhibited comparably by the two antibodies, but U1 snRNA export was only partially inhibited by Ab2. One straightforward interpretation of this result is that the more C-terminal portion of Nup153 that is recognized by Ab2 is not as important for the U1 export pathway as it is for the mRNA and 5S export pathways. Alternately, some other property of Ab2, such as its overall affinity for Nup153, may result in an inefficient block of a Nup153 interaction needed for U1 export. Importantly, in either case, this suggests that Nup153 is either not as critical for U1 export or in fact plays different mechanistic roles in different paths of export.

How do the anti-Nup153 antibodies block export? A concern has been raised previously that any antibodies bound to the nuclear pore complex could cause a nonspecific steric inhibition to the export (or import) of the large transport complexes (Ohno *et al.*, 1998). The results here argue that the Nup153 antibodies in fact have a very specific effect on transport and that this effect is clearly independent of cargo size. For example, Rev protein export is inhibited although this is a small export substrate bound to exportin 1 and the associated transport factor Ran-GTP (Fornerod *et al.*, 1997a). However, the export of the tRNA–exportin-t–Ran-GTP complex, in the same size range as the Rev export complex, is highly efficient in the presence of anti-Nup153 antibodies, as is the export of importin β. The antibodies therefore do not cause general steric interference at the pore but rather selectively disrupt

interactions with Nup153 that are essential to the affected cargo.

One probable candidate for the protein that must interact with Nup153 is the export receptor exportin 1. Exportin 1 (or Crm1) has been identified as a cognate receptor for leucine-rich NESs (Fornerod *et al.*, 1997a; Fukuda *et al.*, 1997; Neville *et al.*, 1997; Ossareh-Nazari *et al.*, 1997; Stade *et al.*, 1997; Ullman *et al.*, 1997). Recent results have indicated that the export of many RNA classes, but not tRNA, is blocked by injection of excess leucine-rich NES sequences (Fischer *et al.*, 1995; Pasquinelli *et al.*, 1997b). This suggests that the 5S RNA, U snRNA, and likely mRNA export pathways use exportin 1. In turn, the anti-Nup153 inhibition results reported here are consistent with a central role for an interaction between exportin and Nup153, an interaction that is disrupted by anti-Nup153 antibodies. However, it remains an open question as to whether an essential interaction of Nup153 and exportin 1 or a different export factor is disrupted by the anti-Nup153 antibodies used here.

The export of tRNA is known to be mediated by a different export receptor, exportin-t (Arts *et al.*, 1998; Hellmuth *et al.*, 1998; Kutay *et al.*, 1998). One would predict from the many differences between tRNA export and the export of mRNA, snRNA, and 5S RNA that exportin-t traverses the pore via a distinct route. This route is clearly not disrupted by Nup153 antibodies (presented here) or by Nup98 antibodies (Powers *et al.*, 1997). Presumably, exportin-t interacts with different nucleoporins. An alternate but interesting possibility is that our Nup153 antibodies do not disrupt some as yet undiscovered interaction of exportin-t with Nup153. Precedence for receptor–Nup153 interactions that are not disrupted by the Nup153 antibodies exists, as discussed above, between importin β and Nup153 (Shah *et al.*, 1998) and between transportin and Nup153 (Shah and Forbes, 1998).

Limited data exist on which subdomains of Nup153 are required for participation in the export of RNA and protein cargos. In an earlier study, Bastos *et al.* (1996) found that overexpression of the FG domain of Nup153 in mammalian tissue culture cells caused nuclear accumulation of poly(A)+ RNA, whereas transfection and expression of the N-terminal or Zn finger domains did not. The observed effect on poly(A)+ RNA accumulation occurred despite the fact that the overexpressed Nup153 FG domain was localized to the cytoplasm. Although this suggested a role for the FG domain in export, because of the complex nature of the transfection approach and the delay before the phenotype could be assessed, it is not clear whether the poly(A)+ RNA accumulation observed was attributable to 1) a block in mRNA export, 2) inhibition of an earlier step in mRNA biogenesis, such as splicing, or 3) an indirect effect caused by blocking a specific, required import pathway. Furthermore, if accumulation was caused by a direct effect on mRNA export, it remained unanswered whether the effect was specific to mRNA or extended to other export substrates. We have now determined that Nup153 is in fact central to U1 snRNA, 5S rRNA, and spliced and intronless mRNA export, as well as to Rev and Rev-mediated RNA export. The role of the FG domain was not specifically assessed in our experiments. Indeed, the inhibitory antibodies Ab1 and Ab2 overlap in their reactivity with the Zn finger domain of Nup153, raising the new possibility that the Zn finger domain contributes to the export function of Nup153. However, because these antibodies may well exert their inhibitory effects by interfering with the function of other domains, detailed functional mapping of Nup153 subregions will be essential in illuminating which Nup153 domain(s) actually interacts with export complexes.

In the nucleus, Nup153 is found on the distal ring of the pore basket and also on intranuclear, pore-associated filaments (Cordes *et al.*, 1993; Sukegawa and Blobel, 1993; Panté *et al.*, 1994). An interesting consideration is whether Nup153 function is different at these two locations. For example, Nup153 localized to the intranuclear filaments might play a role in initiating the export process, whereas basket-associated Nup153 might function primarily in import. The provocative question as to whether such functional distinctions exist between subpopulations of Nup153 molecules is difficult to approach experimentally and awaits the development of specialized techniques for analysis.

To search for clues to the mechanistic role that Nup153 plays in RNA export, we asked whether Nup153 could in fact associate with RNA, either directly or indirectly. Toward this end, we tested whether Nup153 in *Xenopus* egg extracts was able to bind RNA homopolymers. This approach is advantageous for two reasons: 1) an affinity for RNA can be assessed without making assumptions about sequence preference, and 2) the egg extract is likely to contain any adaptor proteins that may be needed to bridge the interaction of export complexes with Nup153. In addition, this approach allowed us to compare Nup153 simultaneously with several other nucleoporins present in the extract. We found that nucleoporins Nup358 and Nup214 did not detectably bind to any of the RNA homopolymers. Nup60 bound marginally over background to poly(G) and poly(U), and low levels of Nup98 bound weakly over background to poly(G) and poly(U). In sharp contrast, Nup153 associated at a high level with poly(G) and to a lesser extent with poly(U). Interestingly, the nonionic detergent NP-40 significantly enhanced the Nup153 binding observed, suggesting that a binding site could be further unmasked under these conditions.

The yeast GLFG nucleoporins Nup145p and Nup116p have been reported to bind poly(G) and

poly(U) (Fabre *et al.*, 1994). The RNP1-like domains thought to confer the RNA-binding property on yeast GLFG nucleoporins are also present in Nup98, the vertebrate homologue of the GLFG family, and are not present in Nup153. Yet, under the conditions examined here Nup153 clearly associates much more tightly with RNA than does Nup98 and does so under more stringent conditions. There exists no obvious homologue of Nup153 for comparison in yeast. It is possible that a role played by the GLFG family of nucleoporins in yeast pores has been assumed by Nup153 in the much larger and complex vertebrate pore. However, even in the case of yeast Nup116p and Nup145p, only a small fraction of the input nucleoporins bound to polyribonucleotide columns (Fabre *et al.*, 1994), similar to the results seen here for the *Xenopus* GLFG protein Nup98 (Figure 7). Thus, it is likely that the GLFG nucleoporins have only a low affinity for RNA in both lower and higher eukaryotes. In contrast, up to ~30% of the Nup153 present in the extract associated with poly(G) under the conditions used here (Figure 7A, lane 2). Nup153 contains several zinc fingers (five in *Xenopus*), which could potentially mediate a direct interaction with RNA. Nup153 has, in fact, been found to interact with DNA in vitro in a Zn-dependent manner (Sukegawa and Blobel, 1993), although the implication of this DNA binding is unclear. Future work will focus on determining whether Nup153 binds to RNA directly or indirectly. At present, however, the RNA binding reveals an important distinguishing property of Nup153.

In this context it is noteworthy that Jarmolowski *et al.* (1994) found that the nuclear injection of poly(G) into oocytes inhibits mRNA, U1, and 5S RNA export, while tRNA export continues normally. In addition, poly(U) was found to have a small inhibitory effect on mRNA export. These homoribopolymers could well inhibit export by disrupting a critical interaction, direct or indirect, between Nup153 and RNA. The homoribopolymers might sequester adaptor proteins that are required to bridge the interaction of RNA and Nup153. Or, if Nup153 interacts directly with RNA, this nucleoporin could itself be the target of inhibitory RNA homopolymers injected into oocytes. However, if Nup153 does interact directly with RNA, it must have additional interactions with receptor–cargo export complexes, because Rev protein export is also inhibited by the Nup153 antibodies.

To date, functional dissection of the vertebrate nuclear pore has been limited. A monoclonal antibody RL1 that recognizes a subset of nucleoporins containing *N*-acetylglucosamine was found to inhibit NLS-directed nuclear import, as well as 5S and tRNA export (Dabauvalle *et al.*, 1988; Featherstone *et al.*, 1988; Terns and Dahlberg, 1994). Wheat germ agglutinin, a lectin that binds to the same set of glycosylated nucleoporins, broadly inhibits both import and export (Finlay *et al.*, 1987; Featherstone *et al.*, 1988; Bataille *et al.*, 1990; Neuman de Vegvar and Dahlberg, 1990). Interestingly, anti-Nup98 antibodies have a strong inhibitory effect on export (Powers *et al.*, 1997), similar to that of the anti-Nup153 antibodies tested here. Specifically, mRNA, U1 snRNA, and 5S RNA export are inhibited by both anti-Nup98 and anti-Nup153 antibodies, while tRNA export and nuclear import take place unabated by the antibodies. These results suggest that Nup153 and Nup98 are functionally linked as components of the export machinery that is used by a subset of important export cargos. However, we have as yet found no indication that the two nucleoporins physically interact with one another. Significantly, the RNA homopolymer-binding experiments indicate that Nup153 and Nup98 are likely to make very different mechanistic contributions to nuclear export. Thus, Nup153 plays an important role as part of the export machinery of the pore and contributes in a specialized manner to the export of multiple export substrates. It is now clear from the results of import studies (Shah and Forbes, 1998) and from the results presented here that multiple pathways of nuclear import and export all converge on the basket of the nuclear pore at Nup153.

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