In Vitro Analysis of Nuclear Transport Mediated by the C-terminal Shuttle Domain of Tap*

The Tap protein of higher eukaryotes is implicated in the nuclear export of type D retroviral mRNA and some cellular mRNAs. Here we have developed an in vitro assay to study nuclear export mediated by the C-terminal shuttle domain of Tap involving the rapamycin-induced attachment of this transport domain to a nuclear green fluorescent protein-containing reporter. We found that export by the Tap transport domain does not involve cytosolic transport factors including the GTPase Ran. The transport domain directly binds to several nucleoporins positioned in different regions of the nuclear pore complex. These results argue that a direct interaction of the Tap transport domain with nucleoporins is responsible for its nucleocytoplasmic translocation. We found that the karyopherin β-related export receptor CRM1 competes with the Tap transport domain for binding to Nup214 but not for binding to Nup62 or Nup153, suggesting that the Tap and CRM1 nuclear export pathways converge at the cytoplasmic periphery of the nuclear pore complex. Because the rates of in vitro nuclear import and export by the Tap transport domain are very similar, the directionality of mRNA export mediated by Tap is probably determined by mechanisms other than simple binding of the Tap transport domain to nucleoporins.

Transport of macromolecules between the nucleus and the cytoplasm occurs through nuclear pore complexes (NPCs), large supramolecular assemblies that span the nuclear envelope (NE) (reviewed in Ref. 1). Although the NPC accommodates passive diffusion of molecules smaller than ~40 kDa, most macromolecules are transported through the NPC by signal- and energy-dependent mechanisms (reviewed in Refs. 2 and 3).

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and 3). In most well characterized cases, nuclear protein import and export is mediated by nucleocytoplasmic shuttling receptors of the karyopherin/importin β family, which are thought to translocate their cargoes by interacting with a series of NPC proteins (nucleoporins) (reviewed in Ref. 2). The best characterized nuclear import pathway involves cargo proteins containing a basic amino acid-rich nuclear localization signal (NLS), which is recognized by the import receptor importin β, either directly or via the adapter importin α. In the most extensively studied nuclear protein export pathway, leucine-rich nuclear export signals (NESs) are recognized by the karyopherin β-related receptor CRM1 (reviewed in Ref. 3).

The small GTPase Ran, which shuttles between the nucleus and the cytoplasm, plays a key role in regulating nuclear transport mediated by karyopherin β-like receptors (reviewed in Refs. 2, 4, and 5). Because of the segregation of the Ran guanine nucleotide exchange factor RCC1 in the nucleus and the Ran GTPase-activating protein in the cytoplasm, the GTP-bound form of Ran is thought to be concentrated in the nucleus and the GDP-bound form in the cytoplasm. RanGTP, which interacts with both import and export receptors, promotes cargo dissociation from import receptors and cargo binding to export receptors in the nucleus (reviewed in Refs. 2 and 3).

Karyopherin β-like receptors are also involved in the nuclear export of tRNAs (6, 7), U small nuclear RNAs (8), ribosomal subunits (9, 10), and certain viral mRNAs (11, 12). On the other hand, the involvement of karyopherin β-related receptors in the export of cellular mRNAs remains uncertain. It is widely believed that proteins bound to mRNAs interact with the nuclear transport machinery to drive export through the NPC (for reviews, see Refs. 4 and 13). Candidate nuclear transport proteins associated with mRNA include heteronuclear RNP A1 (14), Gle2p/Rae1p (15–17), and Tap (18–21), all of which shuttle between the nucleus and the cytoplasm. Tap is a cellular protein that binds to the constitutive transport element of type D retroviral mRNA and mediates export of the latter (22–24). Genetic studies have implicated the yeast homolog of Tap, Mex67p, in the export of some cellular poly(A)+ mRNAs (19, 21), and vertebrate Tap likewise is proposed to have a role in cellular mRNA export (18, 20).

Functional studies identify two different domains of Tap involved in its nuclear transport. A region near the N-terminus binds the karyopherin β-related receptor transportin, which mediates efficient nuclear import of Tap (20, 25). A second region located at the very C terminus (residues 540–619) can mediate nucleocytoplasmic shuttling in and out of the nucleus and is suggested to be critical for the export of mRNA (24, 26). Recent studies have shown that this C-terminal nuclear transport domain (NTD) of Tap can directly bind to Nup214 and Nup98 in vitro (18, 20, 27). Whether this interaction is sufficient for nuclear transport remains unclear. Here we have developed an in vitro assay to study export mediated by the
C-terminal NTD of Tap. Our results indicate that the Tap NTD mediates nuclear export in the absence of Ran and other nucleocytoplasmic shuttling receptors/factors by making direct contact with nucleoporins localized in different regions of the NPC. Furthermore, the NTD promotes nuclear import as efficiently as nuclear export, supporting the notion that the Tap NTD mediates nondirectional translocation through the NPC. The implications of these findings for mRNA export are discussed.

**EXPERIMENTAL PROCEDURES**

**Plasmid Construction**—For the construction of the mammalian expression vector encoding the reporter protein for nuclear export, three additional tandem copies of the FK506-binding protein 12 (FKBP12) were cloned into the Xhol-SalI site of the pBJS vector (a gift from Dr. G. Crabtree (28)) and cloned as a Xhol/BamHI fragment into pTrcHisA. For the FRB-Tap NTD fusion, FRB was amplified by PCR for a Xhol/SacI fragment that introduced a four-residue glycine linker at the C terminus. The Tap NTD (residues 440–619) was amplified by PCR from pFLAG-Tap (a gift from Dr. B. Biesinger), introducing a 5′ SacI and a 3′ SalI site. The FRB and Tap PCR products were cloned into the Xhol/SalI sites of pHTrHisA. To produce an expression vector encoding FRB fused to the mutant Tap NTD A17 (24), a region comprising residues 540–619 of the A17 mutant was obtained by PCR using pTap-A17 (provided by Dr. B. Cullen) as a template. The A17 PCR fragment together with the FRB PCR fragment were cloned into the Xhol/SalI site of pTrcHisA. All constructs contain a hemagglutinin epitope. For immunofluorescence and immunoprecipitation studies, the hemagglutinin tag in these bacterial expression vectors was replaced by the FLAG epitope. The wild type Tap NTD and the Tap A17 NTD were PCR-amplified and introduced as a Sall/EcoRI fragment into the bacterial GST fusion vector pGEX-KG. The accuracy of all constructs was confirmed by DNA sequencing.

Expression and Purification of Recombinant Proteins—6×-His-tagged FRB-Rev NES, FRB-Tap NTD (residues 540–619), FRB-Tap A17 NTD (residues 540–619), and FRB were expressed in E. coli cells and induced at 37 °C and 4 °C to induce the wild type and the Tap A17 NTD were PCR-amplified and introduced as a BamHI/EcoRI fragment into the bacterial GST fusion vector pGEX-KG. The accuracy of all constructs was confirmed by DNA sequencing.

**In Vitro Analysis of Tap Export**

**In Vitro Nuclear Export Assay**—Subconfluent HeLa cells expressing the GFP reporter protein were trypsinized and washed once with 10% fetal calf serum in transport buffer (20 mM Hepes-KOH, pH 7.3, 110 mM potassium acetate, 2 mM magnesium acetate, 1 mM EGTA, 2 mM dithiothreitol, and 1 μM leupeptin, pepstatin, and aprotinin) followed by another washing in another transport buffer containing 10% fetal calf serum. The cell pellets were pelleted with 60 μg/ml digitonin as described previously (34). The permeabilized cells were then preincubated with RanBP1 (15 μg/ml) and an ATP-regenerating system (34) for 5 min at 4 °C following by 3 min at 30 °C to deplete shuttling nuclear transport factors. The standard transport reaction (50 μl in transport buffer) contained an ATP-regenerating system, import ligand (Cys5-LSL-BSA at ~2.5 μM), cytosolic extract (~1 mg/ml), Ran (25 μg/ml), FRB-NESS (~20 ng/ml), and rapamycin (150 ng/ml). Cytosol and import ligand were prepared as described previously (34). For reactions involving ATP depletion, the ATP-regenerating system was replaced by 100 units/ml apyrase (Sigma). For inhibition experiments, the reaction mixture contained either NS2 peptide (44 μg/ml), CRM1 (10 μg/ml), RBDS of Nup358, RanBP2 (90 μg/ml), RanT24N (25 μg/ml), RanQ69L (25 μg/ml), leptomycin B (100 nM), or wheat germ agglutinin (WGA; 150 μg/ml) as indicated. Export reactions were incubated at 30 °C for 25 min and stopped by the addition of ice-cold transport buffer. After centrifugation at 300 × g for 5 min at 4 °C, the cells were resuspended in a small volume of transport buffer, and the average nuclear fluorescence intensity of the GFP reporter protein and Cys5-LSL-BSA in ~10,000 cells was measured using a FACSScan® flow cytometer (Becton and Dickinson).

The monitoring of nuclear export by fluorescence microscopy was performed as described previously (34) except that a Zeiss Axiosvert S100TV inverted microscope interfaced to a Bio-Rad 1024 confocal system was used. Microscopic parameters were identical for each series of acquired images, and digital data were processed identically using Adobe Photoshop (Adobe Systems). To visualize NE staining of the export reporter (Fig. 6), the export reaction was carried out for 5 min and then stopped by adding ice-cold transport buffer. The cells were adsorbed to coverslips, fixed with methanol as described (31), and analyzed as described above.

For monitoring of the diffusion-mediated entry of FRB fusion proteins into the nuclei of permeabilized cells, HeLa cells expressing the GFP reporter protein were grown on coverslips and permeabilized with digitonin as described. The cells were incubated in transport buffer containing 5 mg/ml BSA, FLAG-tagged FRB-NESS proteins, and rapamycin (150 ng/ml). After an incubation of 10 min on ice, cells were washed three times with transport buffer, fixed with 4% formaldehyde, and permeabilized with 0.2% Triton X-100 (31) for 5 min at 4 °C. The cells were then labeled for indirect immunofluorescence microscopy with a rabbit anti-FLAG polyclonal antibody (Sigma) followed by Alexa 594-labeled goat anti-mouse IgG (Molecular Probes).

**Visualization of Nuclear Import**—For real time imaging of nuclear import, GST-Tap NTD (residues 540–619) protein was labeled at a molar ratio of 1:1 with FluorReport Oregon Green 488® (Molecular Probes). The dye was removed by using a Nap5® column (Amersham Pharmacia Biotech). Digitonin-permeabilized HeLa cells grown on glass coverslips were inverted in a reaction mixture in transport buffer containing 5 mg/ml BSA, an ATP-regenerating system, and the labeled GST-Tap NTD (0.4 mg/ml). In some cases, WGA (1 mg/ml) was included or cells were subjected to one cycle of freeze-thawing in liquid N2 before the reaction. Cells were incubated at 20 °C, and nuclear accumulation of fluorescent GST-Tap NTD was monitored by periodic scanning with a confocal microscope. In the time course experiments, images were acquired from different fields of cells for each time point. The average fluorescence intensity was obtained through the histogram function in Adobe Photoshop 5.0. Between 50 and 80 cells from 4–5 independent experiments were counted for each time point, and the standard deviation was calculated from the mean intranuclear fluorescence/extracellular fluorescence ratio. For intranuclear fluorescence determinations, nuclear rim staining was excluded.

**In Vitro Binding Experiments**—NEs were prepared as described (35). 50 μg of NEs were incubated with 100 μg of GST-Tap NTD (residues 540–619), GST-Tap NTD A17 (residues 540–619). To analyze direct binding of recombinant proteins to the GST-Tap NTD, GST-Tap NTD A17 (residues 540–619) was cloned into pGEX-KG and expressed as a GST fusion protein in E. coli cells. The GST fusion protein and cleaved from GST with thrombin. The fragment was purified by anion and cation exchange chromatography.

**Cell Culture and Transfection**—HeLa cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum, 100 units/ml penicillin, and 100 μg/ml streptomycin. Stable HeLa cell lines were obtained by co-transfecting the GFP reporter construct and pCEDNA(−) (Invitrogen) using SuperFect (Qiagen). Approximately 30 G418-resistant clones were pooled and treated overnight with 250 μg trichostatin A (Wako Bioproducts) to increase the expression of the reporter. Cells with high GFP fluorescence were enriched three times by a fluorescence-activated cell sorter (Becton Dickinson). Trichostatin was omitted in the last enrichment step. The nuclear localization of the reporter protein was verified by fluorescence microscopy. The expression of the full-length FKBP-GFP-NLS protein was confirmed by immunoblotting using an anti-hemagglutinin antibody (Berkeley Antibody Co.).
becomes linked to the reporter by rapamycin. In some cases exogenous and export of the reporter is visualized by fluorescence microscopy or therein). Thus, a protein fused to FKBP and a second protein with rapamycin and a recombinant NES-containing protein fused to FRB (FRB-NES). The latter diffuses passively into the nucleus and becomes linked to the reporter by rapamycin. In some cases exogenous cytosol is included in the incubation. Cells are then incubated at 30 °C, and export of the reporter is visualized by fluorescence microscopy or flow cytometry.

bintary nucleoporins to GST fusion proteins, the beads were incubated with the Nup214 fragment (7.5 μg/ml), Nup153 fragment (10 μg/ml), or recombinant Nup62 (10 μg/ml) (each ~ 0.25 μM) in Nonidet P-40 buffer as described above. In the case of Nup62, 10 mg/ml BSA was included in the binding reaction. After incubation for 60 min at 4 °C, the beads were washed 4 times with Nonidet P-40 buffer containing 200 mM NaCl. For competition experiments, beads with bound GST fusion proteins were incubated with CRM1 (75 μg/ml), Ran-GMPPNP (25 μg/ml; pre-loaded as described in Kellenbach et al. (32) or N52 peptide (2 μg/ml) in various combinations as indicated (each ~ 0.8 μM). The incubation and washing was performed as described above except that the Nonidet P-40 buffer contained 30% transport buffer. Bound proteins were analyzed by SDS-polyacrylamide gel electrophoresis followed by immunoblotting or silver staining.

Antibodies, SDS-Polyacrylamide Gel Electrophoresis, and Immunoblotting—Antibodies used for immunoblot analysis were the mouse monoclonal antibodies RL1 and RL2 (36); a rabbit polyclonal antibody against residues 515–537 of rat Nup98, a rabbit polyclonal antibody raised against the C terminus (residues 2073–2090) of human Nup214 (32), a rabbit anti-Nup153 anti-peptide antibody prepared as described in Pante et al. (37) and a rabbit polyclonal antibodies against rat Nup58 and rat Nup62 (33). All polyclonal antibodies were affinity-purified using immunizing antigens. For immunoblot analysis, proteins were separated by SDS-polyacrylamide gel electrophoresis and electrophoretically transferred to nitrocellulose. Blots were blocked overnight incubation with 5% milk powder in TBST (10 mM Tris-HCl, pH 8, 150 mM NaCl, 0.05% Tween 20). Horseradish peroxidase-coupled goat anti-mouse or donkey anti-rabbit IgG (Pierce; 1:10,000 in TBST) were used as secondary antibodies. The enhanced chemiluminescence system (Pierce) was used for visualization of proteins.

RESULTS

In Vitro Export Assay to Study Tap-mediated Nuclear Export—It has been shown previously that the Tap protein shuttles between the nucleus and the cytoplasm (18, 24). A 79-residue stretch at the C terminus of Tap (residues 540–619) can drive the nucleocytoplasmic shuttling of a GST fusion protein (24) and is designated here as the NTD. This Tap region is critical for export of constitutive transport element-containing RNAs, suggesting that it may be key to the export of certain cellular mRNAs (26). We developed an in vitro assay for quantititative biochemical analysis of nuclear export mediated by this Tap region (Fig. 1). Our assay was based on a previously described in vitro assay involving the rapamycin-mediated linking between a reporter protein localized in the nucleus and a NES (28). Rapamycin binds with high affinity (0.4 μM) to the FKBP12 (38). The composite surface formed from this interaction binds strongly to the FRB of FRAP (Ref. 28 and references therein). Thus, a protein fused to FKBP and a second protein fused to FRB can be linked stably by the addition of rapamycin.

Our in vitro export assay involved the use of HeLa cells that were stably transfected with a reporter plasmid coding for four copies of FKBP12 fused to the GFP and the NLS of the SV40 large T antigen (Fig. 1). The reporter, whose size (78 kDa) exceeds the diffusion limit of the NPC, is constitutively localized to the nucleus in vivo. After permeabilization of the cells with digitonin and a preincubation step to deplete shuttling transport factors (see “Experimental Procedures”), cells are incubated in the absence or presence of exogenous cytosol at 0 °C together with rapamycin and a FRB-NES fusion protein. The latter, because of its relatively small size (<30–40 kDa), can enter the nucleus by passive diffusion through the NPC. After a subsequent incubation at 30 °C, the export of the GFP reporter, which is driven by the rapamycin-linked FRB-NES fusion, is analyzed by fluorescence microscopy or flow cytometry.

To analyze Tap-mediated export, we prepared a fusion protein containing FRB attached to the NTD of Tap (residues 540–619). As a control, we made a FRB fusion protein containing the well characterized leucine-rich NES of Rev (9), whose export is mediated by the karyopherin β-related receptor CRM1 (39). The sizes of the Tap and Rev fusion proteins, which were 22 and 15 kDa, respectively, were well below the limit for free diffusion into the nucleus.

When the cells were incubated with the FRB-NES fusions (Rev NES or Tap NTD) in the presence or absence of rapamycin at 0 °C, the FRB-NES fusion proteins became strongly localized to the nucleus only in the presence of rapamycin (Fig. 2A) by rapamycin-induced binding to the nondiffusible FKBP-GFP reporter. The reporter remained localized in the nucleus at 0 °C (Fig. 2A). When the cells were subsequently incubated at 30 °C in the presence of cytosol, strong export of the reporter protein from the nucleus occurred in reactions containing either the Rev NES or the Tap NTD but only in the presence of rapamycin (Fig. 2B). Because the nucleus-released reporter was not associated with cytoplasmic structures (Fig. 2B), we usually used flow cytometry as a rapid method to quantify nuclear export, which was indicated by the loss of cell-associated fluorescence.

A number of controls showed that export mediated by the Rev NES in our assay was physiologically significant. In agreement with the findings from fluorescence microscopy (Figs. 2, A and B, and data not shown), analysis by flow cytometry (Fig. 2C) showed that export mediated by the Rev NES did not occur to a significant level at 0 °C. Moreover, the export seen at 30 °C was dependent on the presence of both exogenous cytosol and rapamycin. The addition of rapamycin usually induced a loss of 50–70% of the fluorescent reporter, although the exact level of export varied from assay to assay (compare export in Figs. 2–4). The dependence of Rev NES-mediated export on exogenous cytosol is consistent with previous findings that preincubation of digitonin-permeabilized cells releases CRM1 and Ran, cytosolic factors that mediate export of leucine-rich NESs (34). Export did not occur with the export-deficient M10 mutant of the Rev NES (40), showing the dependence on a functional NES (data not shown). Furthermore, export was strongly inhibited by incubation with apyrase, which depletes ATP, or by adding the lectin WGA, which binds to the NPC and inhibits receptor-mediated transport (2). The addition of WGA and depletion of ATP also inhibited importin β-mediated import of the fluorescently labeled NLS-BSA that was added as an internal control to the export reactions (data not shown; see “Experimental Procedures”). Considered together, these findings recapitulate previous results showing that in vitro export mediated by a leucine-rich NES is temperature, energy, and cytosol-dependent (34) and validate the physiological significance of this in vitro assay for analysis of signal-mediated nuclear export.

It should be noted that rapamycin alone, in the absence of a

Fig. 1. Schematic diagram of the in vitro nuclear export assay. The assay involves HeLa cells stably transfected with the GFP reporter (4×FKBP-GFP-NLS), which is constitutively localized in the nucleus. The cells are permeabilized with digitonin and then incubated on ice with rapamycin and a recombinant NES-containing protein fused to FRB (FRB-NES). The latter diffuses passively into the nucleus and becomes linked to the reporter by rapamycin. In some cases exogenous cytosol is included in the incubation. Cells are then incubated at 30 °C, and export of the reporter is visualized by fluorescence microscopy or flow cytometry.
FRB fusion protein, induced minimal export of the reporter protein (10–15%) when added to this assay. This effect could be reversed by adding the recombinant FRB domain lacking an attached NES (data not shown). This rapamycin-induced export most likely is caused by a direct or indirect interaction of the FKBP-rapamycin complex with the nuclear export machinery due to the exposed hydrophobic regions of rapamycin, which are sequestered when the FKBP-rapamycin complex is bound by FRB (41).

Export mediated by the Tap NTD in this assay showed some similarity to export obtained with the Rev NES (Figs. 2, A and C, and data not shown). Export by the Tap NTD did not take place when the cells were incubated at 0 °C but occurred efficiently when cells were incubated at 30 °C (i.e. 50–70% of the fluorescent reporter typically was released) (Fig. 2C). Furthermore, export by the Tap NTD was blocked by WGA. It also was specific for a functional export signal, since export was impaired when the wild-type Tap NTD was replaced with the A17 mutant. In this mutant residues 309–595 in the Tap NTD were mutated to alanine, which impaired the nuclear export activity in a binucleate HeLa cell shuttling assay as well as the expression of a Tap-dependent CAT reporter in transfected cells (24).

Export mediated by the Tap NTD as compared with export by the Rev NES (Fig. 2C). First, export by the Tap NTD was only partially blocked by depletion of ATP with apyrase, which may reflect a direct or indirect role of energy in this process. Second, export occurred efficiently in the absence of exogenous cytosol and was not significantly increased by cytosol addition. These data suggest that export mediated by the Tap NTD is not dependent on shuttling nuclear transport factors provided by cytosol, such as CRM1 and Ran.
Export by the Tap NTD Is Independent of CRM1 and Ran—We more rigorously analyzed a possible involvement of CRM1 and Ran in export by the Tap NTD. For this we employed specific conditions to inactivate these components, since it was still possible that small amounts of CRM1 and Ran were not depleted from permeabilized cells in the preincubation step. To investigate a possible CRM1 requirement, we first examined whether a leucine-rich NES peptide derived from the NS2 protein of minute virus of mice (42), which has been shown to interact with CRM1 (43), could compete for Tap-mediated export. Whereas an excess of the NS2 peptide efficiently inhibited nuclear export by the Rev NES in vitro, it had no significant effect on export by the Tap NTD (Fig. 3). We also found that export of the Tap NTD was not inhibited by the drug leptomycin B (Fig. 3), which covalently modifies a critical cysteine residue in CRM1 and inhibits its binding to leucine-rich NESs (44). As expected, leptomycin B strongly inhibited export mediated by the Rev NES (Fig. 3), as reported previously (45). Together these results strongly argue that Tap-mediated nuclear export is independent of CRM1.

We next examined a possible involvement of Ran in nuclear export mediated by the Tap NTD. To deplete RanGTP from the nucleus, we first added an excess of recombinant RBD3 derived from Nup358/RanBP2 (46, 47) to the cells (Fig. 4). This protein region, which is small enough to diffuse into the nucleus, binds strongly to RanGTP (46) and, thus, would be expected to sequester intranuclear RanGTP. As expected, RBD3 inhibited Rev-mediated export (Fig. 4), presumably by preventing the formation of a Rev NES-CRM1-RanGTP export complex. Furthermore, RBD3 strongly inhibited NS2-mediated nuclear import (data not shown). By contrast, RBD3 did not detectably inhibit export by the Tap NTD. In an additional experiment, we added an excess of the Ran mutant RanT24N, which is tightly bound to RanGTP but instead tightly binds to the Ran guanine nucleotide exchange factor (RCC1) (30), thereby inhibiting the formation of endogenous RanGTP. The addition of RanT24N to our assay strongly inhibited export mediated by the Rev NES but had no effect on export by the Tap NTD. Similar results were obtained by adding RBD3 and RanT24N to the cells in the absence of exogenous cytosol (data not shown). Taken together, these results indicate that export mediated by the Tap NTD does not involve CRM1 or Ran. Because all karyopherin β-related nuclear export receptors so far studied appear to form an export complex consisting of RanGTP, NES, and the receptor, the lack of a Ran requirement for export by the Tap NTD indicates that this pathway does not utilize a conventional karyopherin β-related export receptor.

Direct Interaction of the Tap NTD with Nucleoporins—The ability of the Tap NTD to mediate export in the absence of Ran and other soluble shuttling factors could reflect an export mechanism by which the Tap NTD directly interacts with the NPC, as has been proposed to occur for the nuclear import of the human immunodeficiency virus Vpr protein (50–52). Consistent with this possibility, recent studies have shown that the full-length Tap can bind directly to recombinant versions of the nucleoporins Nup214/CAN and Nup98 in vitro (18, 20). We therefore next examined the ability of the NTD of Tap to interact with mammalian nucleoporins in vitro. Initially we carried out in vitro solution binding experiments using solubilized extracts of rat liver NEs. As shown in Fig. 5A, the GST fusion protein containing the Tap NTD (residues 540–619) bound a number of bands ranging from 60 to 300 kDa that were not adsorbed to GST alone or to GST fused to residues 540–619 of the Tap A17 mutant, which was not able to mediate export in our assay (Fig. 2). By immunoblotting, most of these bands were recognized by the RL1 and RL2 monoclonal antibodies (data not shown). These antibodies bind to a group of O-glycosylated nucleoporins containing FG repeats (36), which have been implicated in transport pathways involving karyopherin β-related receptors (reviewed in Ref. 1). Subsequent immunoblotting with specific affinity-purified polyclonal antibodies against a number of the major nucleoporins revealed that Nup214/CAN, Nup153, Nup98, Nup62, and Nup58 were specifically bound to the NTD of wild-type Tap but not to the corresponding region of the A17 mutant (Fig. 5B). The nucleoporins that were identified by this analysis are localized at the nucleoplasmic periphery (Nup153), at the central channel (Nup58 and Nup62) and at the cytoplasmic periphery (Nup214) of the NPC.

To investigate whether or not these binding interactions were direct, we expressed regions of several of these nucleoporins in E. coli and used the corresponding purified recombinant proteins in column binding experiments (Fig. 6A). In particular, we analyzed a C-terminal Nup214/CAN fragment (residues 1861–2090) that comprises most of the segment of Nup214 that was found to interact with Tap in a two-hybrid assay (residues 1805–2090; Ref. 18), the FG-containing C-terminal fragment of human Nup153 (residues 895–1476) and full-length Nup62.
We found that all three of these nucleoporins interacted directly with the wild-type Tap NTD but not with the corresponding region of the A17 mutant (Fig. 6A). Because these nucleoporins are distributed across the NPC, these data support the notion that the Tap NTD can translocate through the NPC by sequential interaction with a series of nucleoporins without the requirement for additional receptor protein(s).

The Binding Sites for the Tap NTD and CRM1 on Nup214 Overlap Functionally—The C-terminal region of Nup214 that binds to the Tap NTD (see the paragraph above) also has been shown to interact with the nuclear export receptor CRM1 (53–55). Furthermore, both Nup153 (56) and Nup62 (32) have been shown to bind to CRM1. This suggests that these nucleoporins may be functionally involved in both the CRM1 and Tap nuclear export pathways. To further investigate this possibility, we examined whether CRM1 could compete for the binding of Nup214 (1861–2090), Nup153 (895–1476), and full-length Nup62 (Fig. 6A) to the Tap NTD. Neither CRM1, Ran loaded with GMPPNP (a nonhydrolyzable GTP analogue), nor the NS2 peptide alone had an effect on the binding of any of these nucleoporins to the Tap NTD (Fig. 6A). By contrast, a combination of CRM1 and Ran-GMPPNP substantially blocked the binding of the Nup214 fragment to the Tap NTD. This inhibition was further increased by the addition of the NS2 peptide. These effects can be explained by previous work showing that RanGTP and the NES peptide cooperatively bind to CRM1 (43) and increase the affinity of CRM1 for certain nucleoporins, including Nup214 (32). In contrast, the same conditions had no effect on the binding of Nup153 (895–1476) or Nup62 (Fig. 6A) to the Tap NTD. This inhibition was further increased by the addition of the NS2 peptide. The strong overlap of the CRM1 and Tap binding sites on Nup214 but are apparently distinct on Nup62 and Nup153.

The strong overlap of the CRM1 and Tap binding site(s) on Nup214 predicts that one of these former two proteins should compete for the export of the other if binding to the C terminus of Nup214 is essential for export. To test this prediction, we carried out in vitro assays to analyze the nuclear export of the Tap NTD in the presence of an excess of the RanQ69L mutant and/or CRM1. Previous work has shown that RanQ69L, a Ran mutant that is deficient in GTP hydrolysis but not in nucleotide exchange (30), inhibits CRM1-mediated export and arrests CRM1 on the cytoplasmic side of the NPC in association with Nup214 (32). In the conditions of our assay, which involved a subsaturating concentration of cytosol, exogenous CRM1 modestly enhanced export mediated by the Rev NES (Fig. 6B). Furthermore, consistent with previous work (32), RanQ69L strongly inhibited export, and this inhibition was further in-
creased by the addition of exogenous CRM1 (Fig. 6B). Whereas a low level of inhibition of export by the Tap NTD was obtained with exogenously added CRM1 and somewhat more inhibition was obtained with RanQ69L, essentially complete transport inhibition was obtained by the addition of an excess of RanQ69L plus CRM1 (Fig. 6B). A similar inhibition of export by the Tap NTD was obtained under the same conditions in assays lacking exogenous cytosol (data not shown). Therefore, these data indicate that the nucleoporin sites occluded by CRM1 binding under these conditions also are required for Tap-mediated nuclear export.

To investigate whether the GFP reporter protein linked to the Tap NTD still became associated with the NPC under the conditions described above, the export reactions were stopped after 5 min of incubation before substantial export of the reporter (and loss of fluorescence) could occur (Fig. 6C). In the control, where the GFP reporter was linked to the Rev NES, the reporter was seen throughout the nucleus in the absence of exogenous CRM1 and RanQ69L, with little or no concentration at the nuclear rim. By contrast, the reporter clearly was present at the nuclear rim (as well as throughout the nuclear interior) in the presence of CRM1 and RanQ69L, presumably reflecting an inhibition of export complex release from the NPC. In contrast, when the GFP reporter was linked to the Tap NTD, the reporter protein was seen in a nuclear rim pattern (as well as throughout the nuclear interior) in both the absence and the presence of exogenous CRM1 and RanQ69L. No nuclear rim concentration occurred when rapamycin was omitted (Fig. 6C and data not shown). These results, which extend our biochemical studies (Fig. 6A), indicate that although the interaction of the Tap NTD with a C-terminal fragment of Nup214 is blocked by CRM1, association with other nucleoporins still occurs.

**Comparison of the Efficiency of Nuclear Import and Export by the Tap NTD**—The Tap NTD mediates nuclear import as well as export (24, 26). Nevertheless, in our in vitro assay the Tap NTD yielded a significant level of net nuclear export (−50–70%). The dominance of nuclear export over nuclear import in this assay may be explained by the high degree of dilution (i.e. up to a thousand-fold) of the GFP reporter-Tap NTD complex once it is released from the nucleus, which would strongly limit its rate of re-import into the nucleus. However, it also is possible that the Tap NTD functions more efficiently to drive nuclear export than import. To distinguish between these possibilities, we carried out real time imaging experiments using a fluorescently labeled GST fusion protein containing the Tap NTD (−68 kDa). After the fluorescent transport substrate was added to permeabilized cells, the ratio of the intranuclear concentration of the fluorescent substrate (NF) to its concentration in the external medium (EF) was followed in real time at different time points.

Visualization of the cells by fluorescence microscopy after 15 min of incubation showed strong nuclear rim staining of the reporter (Fig. 7A). Furthermore, there was a generally uniform intranuclear fluorescence that was similar in intensity to the level of fluorescence in the extracellular medium. The addition of WGA to the cells strongly diminished the nuclear rim labeling and also substantially decreased the level of intranuclear fluorescence (Fig. 7A). This inhibition supports the notion that GFP-Tap NTD crosses the NPC by specific interactions with nucleoporins that are blocked by WGA rather than by passive diffusion, which is not inhibited by WGA (for review, see Ref. 2). Deliberate rupture of the NE by a cycle of freeze-thawing, which allows free diffusion of large proteins into and out of the nucleus, did not result in the concentration of GFP-Tap NTD inside the nucleus. This shows that the fluorescent substrate does not have a significant affinity for intranuclear structures and implies that intranuclear substrate is potentially free to be exported once it enters the nucleus (Fig. 7A). Time course experiments were employed to determine the required time to reach the transport equilibrium that was observed under steady state conditions (Fig. 7A). The fluorescent cargo reached an equilibrium distribution by about 15 min, when the NF/EF ratio was −0.95 (Fig. 7B). This value was similar to the NF/EF ratio at 10 min in freeze-thawed cells (Fig. 7B). These data indicate that the Tap NTD can move in and out of the nucleus at nearly the same rate, which suggests a movement across the NPC without significant directional bias. Thus, even though the binding of the Tap NTD to nucleoporins can specify transport through the NPC, this cannot account for the directionality of mRNA export from the nucleus.

**DISCUSSION**

Here we have developed a novel in vitro assay for biochemical analysis of nuclear export mediated by the Tap NTD, which involves rapamycin-mediated dimerization between a reporter protein and a NES. Using this assay we obtained relatively efficient nuclear export by both the Tap NTD and the previously characterized leucine-rich NES of Rev. We also tested the export ability of the M9 sequence of heteronuclear RNP A1 (14) and the KNS sequence of heteronuclear RNP K (57) in our assay, because both sequences have been reported to have nucleocytoplasmic shuttling activity in vivo. However, in our assay neither M9 nor KNS showed any export activity.2 This

2 I. Schmitt and L. Gerace, unpublished observations.
may be due to the fact that they are weak NESs (58) or the possibility that they mediate export indirectly by interacting with other heterogeneous nuclear RNP particles, which probably would not be detected in our assay.

Surprisingly, comparison of the Tap NTD and the Rev NES revealed significant differences between them. The Tap NTD, unlike the Rev NES, does not depend on shuttling cytosolic factors including CRM1 and Ran. Although our preincubation procedure depletes shuttling factors quite efficiently (32), additional evidence that RanGTP is not involved in export by the Tap NTD was provided by the finding that export is not affected by RanT24N or RanBD3. Thus, our analysis provides direct functional evidence that karyopherin β-related export receptors are not involved in this pathway, since all receptors of this class that have been characterized so far require RanGTP for export.

Our binding studies with recombinant proteins showed that the Tap NTD directly interacts with several nucleoporins, which are localized at the cytoplasmic periphery (Nup214), the nucleoplasmic periphery (Nup153), or in the central region (Nup62) of the NPC. Furthermore, this binding is not dissociated or enhanced by Ran in the GTP bound state, unlike the binding of karyopherin β-like receptors to most nucleoporins, which is modulated by RanGTP (32, 43, 59, 60). Our results extend previous studies that reported direct Tap binding to a fragment of the nucleoplasmic nucleoporin Nup98 and to the C-terminal region of Nup214 (18, 20). Considered together, these nucleoporin binding studies suggest that Tap may be translocated through the NPC by sequentially binding to a series of different nucleoporin binding sites at the NPC, as proposed for the translocation of karyopherin β-like receptors (33).

It should be noted that we observed strong in vitro binding of all of the nucleoporins tested to the Tap region comprising residues 540–619. Moreover, no increase in the efficiency of nucleoporin interaction was obtained with a Tap fragment comprising residues 352–619. Consistent with these results, this Tap region (residues 540–619) contains the region that is important for nucleoporin binding based on deletion analysis (27) and point mutagenesis (24). Nevertheless, it is possible that larger regions of Tap could have an increased nucleoporin affinity that is undetectable by our methods (compare with Ref. 20).

Interestingly we found that CRM1 does not block the binding of the Tap NTD to Nup62 (localized in the central channel) and the FG repeat-containing fragment of Nup153 (localized in the nucleoplasmic fibrils). In contrast, CRM1 strongly inhibited the association with the Nup214 C-terminal fragment (localized in the cytoplasmic fibrils), thus indicating overlapping binding sites for CRM1 and Tap on Nup214. Correspondingly, preincubation of the permeabilized cells with CRM1 and the RanQ69L mutant, which causes arrest of CRM1 at Nup214 (32), also causes inhibition of export by the Tap NTD. However, these conditions do not inhibit NE association of the Tap NTD, presumably because it can still interact with more proximal NPC binding sites. Consistent with this, the co-immunoprecipitation of Nup153 with the Tap NTD in solubilized cell extracts from our export assay was undiminished after permeabilized cells were preincubated with CRM1 and RanQ69L.2 It is intriguing to suggest that the CRM1 and Tap NTD export pathways may converge at Nup214, making this protein a potential regulatory hub for nuclear export.

One of the key features of both receptor-mediated nuclear protein export and mRNA export is its highly directional (nuclear-to-cytoplasmic) nature. Because the Tap NTD is suggested to be important for the export of some mRNAs (26), we examined whether this sequence drives transport through the NPC with strong bias in the nuclear-to-cytoplasmic direction. Interestingly, we found that in the absence of cytosol and shuttling factors, the Tap NTD (fused to a large carrier protein) was imported into the nucleus of permeabilized cells as efficiently as it was exported, rapidly reaching a steady state nuclear/cytoplasmic concentration ratio of about 1. In cells, Tap is much more concentrated in the nucleus than in the cytoplasm (18), probably because of the presence of a NLS recognized by transportin in the N-terminal region of Tap that specifies its directional import (20, 25). In addition, there may be intranuclear binding sites for Tap that promote its nuclear retention, which are absent from the NTD.

Although this work indicates that the NTD does not have directional bias in its movement through the NPC, it remains possible that another region of Tap helps to specify directional export of Tap out of the nucleus. Tap contains a binding site in residues 352–550 for p15/NXT1 (18), a RanGTP-binding protein that stimulates the export of a leucine-rich NES in vitro (61, 62). Functional studies suggest that NXT1 acts by promoting the binding of Tap to the constitutive transport element (26). In our assay, however, a larger region of Tap containing the NXT1 binding site (residues 352–619) was not able to mediate nuclear export. Also including cytosol and exogenous NXT1 that was active in promoting Rev-mediated export did not stimulate export of this Tap version.2 At the level of messenger RNA export, it is plausible that the cytosolic RNA helicase Dhp5 (63–65) or other components that restructure messenger RNPs as they exit the nucleus (for review, see Ref. 66) contribute to the directionality of transport. In this model, the nuclear export of messenger RNA particles would be driven by a combination of several different transport elements such as the Tap NTD that we have analyzed here. In this respect mRNA export would be significantly more complex than the translocation of proteins through the NPC.

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