RNA Export Mediated by Tap Involves NXT1-dependent Interactions with the Nuclear Pore Complex*

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Nuclear export of ribonucleoprotein complexes requires cis-acting signals and recognition by receptors that mediate translocation through the nuclear pore complex. Translocation is likely to involve a series of physical interactions between the ribonucleoprotein complex and nucleoporins within the nuclear pore complex. Here, we have characterized the function of NXT1 in the context of the Tap-dependent RNA export pathway. Tap has been implicated in the nuclear export of RNA transcripts derived from Mason-Pfizer monkey virus that contain the constitutive transport element. We demonstrate that NXT1 stimulates binding of a Tap-RNA complex to nucleoporins in vitro, and we provide mutational analysis that shows these interactions are necessary for nuclear export of an intron-containing viral mRNA in vivo. Tap contains separate domains for binding to nucleoporins and NXT1, both of which are critical for its export function. RNA export is mediated by a heterodimer of Tap and NXT1, and the function of NXT1 on this pathway is to regulate the affinity of the Tap-RNA complex for nucleoporins within the nuclear pore complex. We propose that NXT1-dependent binding of the Tap-RNA complex to the nucleoparin p62, which we have reconstituted in vitro using recombinant proteins, represents a single step of the translocation reaction.

Movement of protein and RNA between the nucleus and the cytoplasm involves facilitated transport through the nuclear pore complex (NPC). Both import and export pathways rely on receptors that recognize cis-acting transport signals within protein or RNA cargo. Receptors and cargo assemble, translocate through the NPC, and disassemble at the end of the transport reaction (1, 2). Substantial effort has been applied to gain an understanding of the molecular basis of these transport events, including how these events may be regulated. It is well established that components of the NPC, termed nucleoporins, are directly involved in transport. Nucleoporins, in addition to contributing to the structural architecture of the NPC, provide binding sites for receptor-cargo complexes during nuclear transport and for cargo-free receptors during recycling. Current evidence suggests that the receptor-binding sites within nucleoporins include peptide repeats that are based on the motif FXFG or GLFG, referred to as FG repeats (3, 4). Conceptually, these binding sites on nucleoporins could be used for the highly transient interactions expected to occur during the translocation reaction and for assembly or disassembly reactions that initiate or terminate transport, respectively. As yet, there is no evidence that transport through the NPC involves a force-producing mechanism. Rather, the cumulative work from many laboratories is consistent with nuclear transport occurring through a series of receptor-nucleoporin interactions, the directionality of which is probably determined by binding affinities.

Biochemical and genetic approaches have been used to identify nuclear transport receptors in a variety of organisms. Most of the receptors studied to date are members of the β-importin superfamily (1, 2). The founding member is β-importin (also termed β-karyopherin). β-Importin, generally with the assistance of an adapter protein termed α-importin (also termed α-karyopherin), binds nuclear localization signal-containing proteins and mediates import into the nucleus. The most thoroughly characterized export receptor, Crm1, binds directly to nuclear export signal (NES)-containing proteins and mediates their export to the cytoplasm. Certain transport pathways, however, appear to operate independently of β-importin family members. For example, nuclear export of the glucocorticoid receptor does not rely on Crm1 but instead uses calreticulin to mediate its delivery to the cytoplasm (5).

Nuclear export of different classes of RNA occurs on several pathways, which reflects the use of distinct receptors and, in some cases, pathway-specific adapters or regulators. The receptor Crm1, which mediates nuclear protein export, can also mediate RNA export. This requires NES-containing adapter proteins such as Rev, which bridges the interaction between Crm1 and HIV-1 RNA. PHAX bridges the interaction between Crm1 and U1 small nuclear RNA (6), and Nmd3p bridges the interaction between Crm1 and the 60S ribosomal subunit (7). Nuclear export of tRNA, which does not require an adapter protein, is mediated by the β-importin superfamily member transportin-1 (8).

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‡The abbreviations used are: NPC, nuclear pore complex; BSA, bovine serum albumin; CTE, constitutive transport element; IF, immunofluorescence; IP, immunoprecipitation; LSC, liquid scintillation counting; MPMV, Mason-Pfizer monkey virus; NES, nuclear export signal; PAGE, polyacrylamide gel electrophoresis; PBS, phosphate-buffered saline; RRE, Rev response element; WT, wild type; GST, glutathione S-transferase; HIV-1, human immunodeficiency virus, type 1.
Establishing the identities of receptors that are directly responsible for mRNA export has proven more difficult, perhaps because of the heterogeneous nature of the RNA cargo. Moreover, there is relatively little information on cis-acting export signals in cellular mRNAs. However, cis-acting export signals present in certain viral RNAs have been characterized extensively, and the analysis of these signals has provided general insights into RNA export mechanisms (9). The Rev response element (RRE) is a 234-nucleotide sequence within transcripts derived from HIV-1 that forms a stem-loop structure (10, 11). The RRE provides the binding site for the viral protein Rev that, through its NES, can target the RNA for export on the Crm1 pathway (12). The constitutive transport element (CTE) is a 183-nucleotide sequence present in type D retroviral transcripts such as those from Mason-Pfizer Monkey virus (MPMV; see Ref. 13). The CTE is thought to be functionally similar to the RRE, despite the lack of sequence relatedness between the two signals (14, 15). Unlike HIV-1, the genome of MPMV does not encode a RNA-binding protein analogous to Rev. Instead, MPMV and other type D retroviruses rely on the host cell machinery to recognize the CTE and mediate transport of the CTE-containing RNA transcripts to the cytoplasm (9).

The host cell factor that has been suggested to recognize and mediate nuclear export of CTE RNA is Tap (16). The Tap protein binds directly to the CTE through its amino-terminal domain, and microinjection or transfection of Tap promotes nuclear export of CTE-containing RNA in oocytes (16, 17) and in quail cells (18). These results, together with the fact that Tap interacts with the NPC and shuttles between the nucleus and cytoplasm (18–20), suggest that Tap may function as a receptor for RNA export. Tap activity is regulated by a nuclear export factor termed NXT1 (21–24). The connection between Tap and NXT1 came initially from two observations by the Hurt laboratory. First, NXT1 (also called p15) was detected as a polyepitope in a co-IP with Tap from a mammalian cell extract (22). Second, co-expression of NXT1 and Tap was found to partially rescue growth in a Saccharomyces cerevisiae strain with mutations in Mex67 and Mtr2, the gene products of which are required for mRNA export (22, 25, 26). We identified NXT1 based on its sequence relatedness to the nuclear import factor NTF2 and have shown that it functions on the Crm1-dependent export pathway for NES-containing proteins (21). NXT1 binds to the Crm1 export complex and together with RanBP1 facilitates export complex disassembly and release from the cytoplasmic side of the NPC (27).

The function of NXT1 in Tap-dependent RNA export has not been defined, and its involvement in this pathway has been somewhat controversial. CTE RNA export in the oocyte can be stimulated by a mutant form of Tap with a carboxyl-terminal deletion that removes the NPC and NXT1 binding domains (17). A NXT1-binding mutant of Tap that contains three alanine substitutions (amino acids 415–417) can still stimulate CTE RNA export, albeit at a level that is ~50% of the wild type (WT) Tap (23). In contrast to these results suggesting that NXT1 plays only a minor role in export, we found that Tap-dependent export of RNA is strongly dependent on NXT1 (24). Our analysis was based on an RRE-containing reporter RNA and the nuclear export of this transcript by an export-defective mutant of Rev (RevM10) that is fused to Tap. In the context of this assay, RevM10 provides the RNA binding function and Tap provides the transport function. Co-translation of NXT1 stimulates Tap-dependent RNA export up to 40-fold in vitro, and it is strictly dependent on a functional NXT1 binding domain in Tap (24). This suggested that NXT1 is a critical co-factor for Tap-dependent RNA export.

We set out to determine the function of NXT1 in Tap-dependent RNA export in mammalian cells. We found that NXT1 increases the affinity of Tap for nucleoporins up to 6-fold in vitro. This observation, together with our finding that NXT1 stimulates Tap-dependent RNA export (24), suggests that nuclear export on this pathway is directly linked to the affinity of Tap for nucleoporins within the NPC. Tap binds to p62 by both NXT1-independent and NXT1-dependent mechanisms. Both forms of binding require the carboxyl-terminal domain of Tap and correlate with the ability of Tap to mediate RNA export. A simple ribonucleoprotein complex containing Tap and CTE RNA binds to the nucleoporin p62 in a reaction that is stimulated by NXT1. Thus, NXT1 can regulate the interaction of a Tap-RNA export complex with the NPC.

**EXPERIMENTAL PROCEDURES**

Recombinant Protein Expression and Purification—Recombinant NXT1 (WT or mutant) and NTF2 were expressed from pET plasmids in the BL21(DE3) strain of *Escherichia coli* as described previously (21, 27, 28). The pGEX plasmids encoding GST-p62 and GST-p58 and the vectors derived from GST-p58 (10, 11, 29) were kindly provided by Dr. Larry Gerace (29). The Tap cDNA (GenBank™ accession number AA173362) was amplified by polymerase chain reaction and cloned into the vector pGEX-4T3 plasmid (Amersham Pharmacia Biotech). The GST fusions of nucleoporins p62 and p58, and Tap were expressed in *E. coli* by isopropyl β-D-thiogalactoside induction and isolated on glutathione-Sepharose.

*In Vitro Transcription and Translation—*The [35S]methionine-labeled proteins used in this study were prepared with the coupled transcription/translation system using rabbit reticulocyte lysate (Promega) and plasmid templates. The [35S]methionine used had a specific activity of 1175 Ci/mmol (TransLabel, ICN). The incorporation of [35S]methionine into protein was measured by filter binding to 0.45-μm HAWP nitrocellulose membranes, which was followed by extensive washing with liquid scintillation counting (LSC). The specific activity of [35S]methionine-labeled proteins took into account the fact that rabbit reticulocyte lysates contain 5 μM methionine, based on the recommendation of the supplier (Promega). The Tap cDNA (GenBank™ accession number AA173362) was amplified by polymerase chain reaction and cloned into the vector pcDNA3.1 (Invitrogen) that was modified to contain an amino-terminal FLAG tag. The Rev and GAG polyclams (WT and deletion mutants) have been described previously (24). The FLAG-NXT1 plasmid has also been described (21). Point mutations (N48E, N48K,N50K, E102N, R107A, K127A) were introduced into the FLAG-NXT1 plasmid using the QuickChange site-directed mutagenesis system (Stratagene) and confirmed by DNA sequencing. A plasmid encoding NXT1 that lacks a fusion tag was generated by subcloning the open reading frame of mouse NXT1 into the mammalian expression vector pSVK3 (Amersham Pharmacia Biotech).

The [32P]UTP- and [32P]GTP-labeled CTE RNAs were synthesized using pGEM-based templates and T7 polymerase. The WT CTE (pGEM2zfCTEwt) and M2/M11 mutant CTE (pGEM2zfCTEM2) plasmids (30) were linearized with *Bam*HI, and the GAC mutant CTE (pGEM2zfCTEM2) plasmid (18) was linearized with *Xho*I. The transcription reactions contained [α-32P]UTP (ICN) or [α-32P]GTP (PerkinElmer Life Sciences) with a specific activity of 800 Ci/mmol and unlabeled DNTPs (Promega). Radiolabeled RNA was heat-denatured at 93 °C for 3 min, fractionated on a 6% polyacrylamide gel containing 7 M urea, and isolated by the crush and soak method. Renatured RNA was analyzed on 6% native polyacrylamide gels prior to use in binding assays. The plasmid encoding the GAC mutant (mutGAC) of the CTE was kindly provided by Dr. Bryan Cullen.

**Immunoprecipitation and GST Pull-down Assays—**NXT1 binding was examined by co-immunoprecipitation (IP) of [35S]methionine-labeled proteins out of rabbit reticulocyte lysate. FLAG-Tap and untagged NXT1 (WT and mutant) were synthesized separately as [35S]methionine-labeled proteins. Each binding reaction (20 μl total volume) contained 2 μl of lysate from the in vitro transcription/translation reactions, bovine serum albumin (BSA, 0.1 mg/ml), aprotinin, leupeptin, and pepstatin (each at 2 μg/ml), phenylmethylsulfonyl fluoride (0.5 mM), and dithiothreitol (2 mM) in phosphate-buffered saline (PBS: 137 mM NaCl, 2.7 mM KCl, 10 mM Na HPO4, 1.76 mM KH2PO4, pH 7.4). The binding reactions were incubated for 1 h at 4 °C prior to the addition of antibodies. For IP, the FLAG monoclonal antibody M2 was immobilized
**Fig. 1.** Localization of NXT1 and Tap to the cytoplasmic side of the NPC by fluorescence microscopy. A, cell lines expressing FLAG-NXT1 or FLAG-Tap were permeabilized with (0.005%) digitonin followed by formaldehyde fixation and were processed for IF without further permeabilization; these conditions are known to preserve nuclear membrane integrity and only expose epitopes present on the cytoplasmic face of the nuclear pores. NXT1 and Tap were visualized using α-FLAG antibodies (middle and right panels, respectively), and the nucleoporin RL1 antibody was used to reveal nuclear pores (left panel). Scale bars = 2 μm. B, localization of NXT1 to the cytoplasmic and nucleoplasmic sides of the NPC by immuno-EM. Gallery of images showing the nuclear envelope membranes from a HeLa cell line expressing FLAG-NXT1 that was processed for immuno-EM. FLAG-NXT1 (arrows) was detected using anti-FLAG and 10 nm gold-conjugated anti-mouse antibodies. The cytoplasmic (c) and nuclear (n) compartments are indicated. The scale bar = 0.2 μm. C and D, distribution of NXT1 plotted as the percentage of gold particles and distance from the central plane of the NPC. The average distribution of NXT1 is ~40 nm from the central plane on the two sides of the NPC.
on protein G beads (both from Sigma) at a concentration of 0.2 μg of antibody per μl of packed beads. The antibody beads were mixed with the binding reaction overnight at 4 °C. The immunoprecipitates were washed six times with PBS containing 0.1% Nonidet P-40. The proteins bound by the FLAG antibody were eluted by boiling the beads with SDS sample buffer, resolved by SDS-polyacrylamide gel electrophoresis (PAGE), and visualized by autoradiography. For the GST pull-down assays, the lysates were diluted as above and mixed with GST or GST-p62 immobilized on glutathione beads (20 μl of beads containing 10 μg of protein) overnight at 4 °C. The beads were washed and processed as described for the IP analysis. In the case of the RNA co-IP experiments, the reactions also contained 32P-labeled RNA (4.3 × 105 cpm/binding reaction), unlabeled tRNA (20 μg/ml), and ribonuclease inhibitor (10 units/ml; Life Technologies, Inc.), GST-Tap (400 ng) or GST (5 μg). Levels of precipitated [32P]-CTE RNA were quantitated by LSC.

Microtiter Well Binding Assays—The solid phase binding assays were performed essentially as described (21). The GST fusion proteins (~30 nm final concentration) were diluted into transport buffer (20 mM Hepes, pH 7.4, 110 mM potassium acetate, 2 mM magnesium acetate, 1 mM EGTA) and adsorbed to the wells (100 μl/well) of high capacity protein-binding 96-well plates (Costar catalog number 3590) for 24 h at 4 °C. The wells were then blocked with 3% BSA (300 μl) for 24 h at 4 °C. The input for the binding reactions were assembled in microcentrifuge tubes and contained [35S]methionine-labeled FLAG-Tap (~105 cpm/well), 0.5% BSA, 0.2% Tween 20, and 2 mM dithiothreitol in transport buffer, in the presence or absence of recombinant NXT1 or NTF2. For RNA-binding experiments, the reactions also contained [35S]methionine-labeled FLAG-Tap (~105 cpm/well), unlabeled tRNA (14 μg/ml), and ribonuclease inhibitor (14.3 units/ml; Life Technologies, Inc.). After binding for 24 h at 4 °C, the wells were washed three times in 0.5× transport buffer containing 0.2% Tween 20, and eluted with 5% SDS (200 μl) for 30 min at room temperature. The level of [35S]methionine-labeled FLAG-Tap and [35P]UTP-labeled RNA was measured by LSC in separate channels. We determined that 30% of counts from [35S] appear in the window for [35S] (data not shown), making it necessary to correct for [35S]-protein binding. In assays using only recombinant factors, GST-Tap (140 ng/well) was used instead of reticulocyte lysate-translated FLAG-Tap, and soluble GST (400 ng/well) was added to all binding reactions.

Microscopy—HeLa cells expressing FLAG-NXT1 (21) or FLAG-Tap grown on glass coverslips were used for immunofluorescence (IF) microscopy. HeLa cells expressing FLAG-Tap were generated by electroporation and processed for IF 48 h post-transfection. For selective detection of NXT1 and Tap on the cytoplasmic side of the NPC, the cells were treated with 0.005% digitonin in transport buffer for 6 min on ice, washed in transport buffer, fixed in 2% formaldehyde, and processed for

Fig. 2. NXT1 regulates Tap binding to nucleoporins. A, Tap and NXT1 binding to GST-p62 (lanes 1–3) or GST (lanes 4–6) immobilized on glutathione beads. Binding reactions were performed in PBS with BSA as a carrier protein and contained 35S-labeled Tap and/or NXT1 as indicated. Bound fractions were analyzed by SDS-PAGE and autoradiography. B, NXT1 stimulates Tap binding to p62. Microtiter well binding assays were performed using GST-p62 (●), GST (□), or BSA (●) adsorbed to the wells of high binding capacity, 96-well plates. 35S-Tap prepared in reticulocyte lysate was added to each well in the absence or presence of NXT1. 35S-Tap binding was plotted as the mean of triplicate (±S.E.) of three replicates. D, Tap and NXT1 interactions with specific domains of nucleoporin p58. GST fusions of full-length p58 and three deletion derivatives (29) spanning the amino (GST-p58N; amino acids 1–225), middle (GST-p58M; amino acids 226–410), and carboxyl (GST-p58C; amino acids 411–585)-terminal domains were adsorbed to microtiter wells and tested for 35S-Tap binding in the absence (●) and presence (□) of NXT1. Tap binding was expressed as mean fmol (±S.E.) of three replicates.

B

C

D

[Image]

on protein G beads (both from Sigma) at a concentration of 0.2 μg of antibody per μl of packed beads. The antibody beads were mixed with the binding reaction overnight at 4 °C. The immunoprecipitates were washed six times with PBS containing 0.1% Nonidet P-40. The proteins bound by the FLAG antibody were eluted by boiling the beads with SDS sample buffer, resolved by SDS-polyacrylamide gel electrophoresis (PAGE), and visualized by autoradiography. For the GST pull-down assays, the lysates were diluted as above and mixed with GST or GST-p62 immobilized on glutathione beads (20 μl of beads containing 10 μg of protein) overnight at 4 °C. The beads were washed and processed as described for the IP analysis. In the case of the RNA co-IP experiments, the reactions also contained 32P-labeled RNA (4.3 × 105 cpm/binding reaction), unlabeled tRNA (20 μg/ml), and ribonuclease inhibitor (10 units/ml; Life Technologies, Inc.), GST-Tap (400 ng) or GST (5 μg). Levels of precipitated [32P]-CTE RNA were quantitated by LSC.

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represents fmol of $^{35}$S-Tap bound as the mean (±S.E.) of three replicates. Each point and presence (black) was assayed as a function of the concentration of Tap, both in the absence (circles) and presence (squares) of NXT1. One point represents fmol of $^{35}$S-Tap bound as the mean (±S.E.) of three replicates.

IF without further permeabilization as described by Black et al. (21). The antibodies used were the anti-FLAG monoclonal M2 (1:5000, Sigma) followed by a Cy3-conjugated anti-mouse (1:200, The Jackson Laboratories) and the nucleoporin antibody RL1 (1:100, see Ref. 31) followed by rhodamine-conjugated anti-mouse IgM (1:200, Pierce). Epi-fluorescence images were captured digitally on a Nikon microphot SA microscope and processed using Adobe Photoshop (version 5.5). The RL1 antibody was kindly provided by Dr. Larry Gerace. The methods used for immunoelectron microscopy (immuno-EM) localization have been described (32). The average distribution of NXT1 was determined by measuring the distance between gold particles and the central plane of the NPC.

RNA Export Assay—The methods used to assay RNA export in 293T/17 cells were recently described in detail (24). The assay measures nuclear export of a transcript encoding the HIV-1 capsid protein p24 (33), the expression of which is quantitated using a commercially available enzyme-linked immunosorbent assay (PerkinElmer Life Sciences). Nuclear export of the HIV-1 transcript is dependent on the functional domains of Tap based on published data (19, 20, 23, 24). The RevM10 domain mediates binding of the Tap fusion to the RRE in the HIV-1 RNA. The CTE binding domain includes four leucine-rich repeats (LRR) (20). The domains for NXT1 and NPC binding, which appear to have partial overlap, are also indicated. B, analysis of Tap deletion mutants with respect to NXT1-dependent and NXT1-independent binding to nucleoporin p62. The FLAG-RevM10-Tap proteins (WT and mutant) were translated in vitro as $^{35}$S-methionine-labeled proteins and used in microtiter well binding assays with immobilized GST-p62 (black) or GST (gray). Binding of Tap was expressed as mean (±S.E.) of four replicates.

RESULTS

NXT1 and Tap Localization at the NPC—Previous work (21, 22) has established that NXT1 localizes to both the nucleoplasm and the NPC, and similar results have been obtained with Tap (19, 20). To determine whether NXT1 and Tap are associated specifically with the cytoplasmic face of the NPC, HeLa cells expressing FLAG-NXT1 or FLAG-Tap were treated with a concentration of digitonin that permeabilizes the plasma membrane and leaves the nuclear membrane intact. Following a brief fixation, we processed the cells for IF microscopy using an anti-FLAG antibody to detect transfected NXT1 or Tap and RL1 antibody to detect NPCs. Under these conditions, the primary and secondary antibodies have access to the cytoplasmic compartment but not to the nucleoplasmic compartment (27). We observed a punctate distribution of NXT1 and Tap on the cytoplasmic side of the nuclear envelope that was very similar to the distribution of NPCs detected with RL1 (Fig. 1A). Thus, under steady state conditions, NXT1 and Tap are localized to the cytoplasmic side of the NPC.

We examined the subcellular distribution of NXT1 using immuno-EM. The FLAG-NXT1 cell line was processed for immuno-EM using the anti-FLAG primary and 10 nm gold-labeled secondary antibodies. NXT1 localized to both the cytoplasmic and nuclear sides of the NPC (Fig. 1B). We quantitated the distribution of NXT1 by measuring the distance between individual gold particles and the central plane of the NPC. This analysis revealed that NXT1 localizes ~40 nm from the central plane of the NPC on both sides of the pore (Fig. 1, C and D).

NXT1 and Tap Binding to Nucleoporins—The interaction of Tap with the NPC is predicted to be fundamental for its role in mediating translocation of RNA substrates through the pore. Deletion mutants of Tap, analyzed by subcellular localization and binding experiments using individual nucleoporins, have implicated the carboxyl-terminal ~100 amino acids (residues 508–619) as important for interactions with the NPC (19, 20). Because NXT1 also localizes to the NPC and is essential for Tap-dependent export of RNA (24), we hypothesized that NXT1 might regulate the interaction of Tap with the NPC. We initiated this analysis using p62, a nucleoporin that is known to bind Tap and several other nuclear transport factors (20, 28, 29). We first tested whether NXT1 and Tap synthesized in reticulocyte lysate as $^{35}$S-labeled proteins could bind to GST-p62. In separate binding reactions, both NXT1 and Tap bound to GST-p62 but not to the GST control (Fig. 2A). Both Tap and NXT1 binding to p62 were increased when the reaction contained both $^{35}$S-methionine-labeled proteins (lane 3). This result provided the first clue that Tap and NXT1 might display cooperative binding to the NPC.

To quantitate the interactions between Tap, NXT1, and p62, we used a solid phase binding assay with GST-p62 adsorbed to
addition of recombinant NXT1 (63 nM final), whereas addition binding to p62 was stimulated approximately 6-fold by the S.E. plotted as the mean of three values (H11006 uH11006) using the microtiter well binding assay. Levels of Tap binding were tested for their ability to stimulate Tap binding to GST-p62 (o or GSTfbinant WT and mutant NXT1 proteins expressed in bacteria were and autoradiography. B, NXT1 binding to nucleoporin p62. GST-p62 (lanes 1–6) and GST proteins (lanes 7–12) immobilized on glutathione beads were combined with [35S]methionine-labeled WT or mutant NXT1. The bound fractions were analyzed by SDS-PAGE and autoradiography. C, activity of NXT1 proteins in Tap binding to p62. Recombinant WT and mutant NXT1 proteins expressed in bacteria were tested for their ability to stimulate Tap binding to GST-p62 (■) or GST (□) using the microtiter well binding assay. Levels of Tap binding were plotted as the mean of three values (±S.E.).

microtiter wells, to which we added [35S]methionine-labeled Tap in the absence and presence of NXT1. We found that Tap bound specifically to GST-p62 (0.712 ± 0.009 fmol) and displayed a low level of background binding to GST (0.246 ± 0.009 fmol) and BSA (0.192 ± 0.017 fmol) (Fig. 2B). Strikingly, Tap binding to p62 was stimulated approximately 6-fold by the addition of recombinant NXT1 (63 nM final), whereas addition of the same concentration of NTF2 had no effect (Fig. 2B). We performed the binding assay over a broad concentration range of recombinant NXT1 (Fig. 2C). We observed a maximal level of Tap binding to p62 at a concentration of ~100 nM NXT1. We obtained similar results using nucleoporins that are components of the p62 complex (32), including p58 (Fig. 2D) and p54 (data not shown). Tap bound to both the amino- and carboxy-terminal domains of p58 in NXT1-independent and NXT1-dependent reactions (Fig. 2D). In contrast, Tap did not bind to the middle domain of p58. The amino- and carboxy-terminal domains of p58, but not the middle domain of p58, contain multiple FXFG motifs and bind to the nuclear import factors NTF2 and β-importin (29). Thus, the same domains of p58 may be used as binding sites for both the import and export machinery. We also found that Tap binding to p62 is saturable in the absence and presence of NXT1 (Fig. 3). This could be explained by different binding states of Tap, which are NXT1-independent and NXT1-dependent. Alternatively, NXT1 may modulate Tap binding to a class of higher affinity binding sites on p62.

Our finding that NXT1 stimulates Tap binding to p62 was highly interesting in light of previous structure-function studies that have revealed the domain organization of Tap (Fig. 4A). In particular, the carboxyl-terminal region of Tap contains binding sites for both the NPC and NXT1 (19, 20, 22, 24). Deletion of only nine amino acids from the carboxyl terminus reduces binding to p62 (20). The NXT1 binding domain of Tap has been mapped to an ~180-amino acid domain (residues 372–551) that partially overlaps the NPC binding domain (20). Deletion of only 34 amino acids within this region of Tap (Δ507–540) abolishes binding to NXT1 (24). Because of the close proximity of the NXT1- and NPC-binding sites on Tap, we hypothesized that NXT1 binding might induce a conformational change in the carboxyl terminus of Tap that increases its affinity for p62. We also considered the possibility that NXT1 bridges the interaction between Tap and p62 and that the Tap-NXT1-p62 complex is more stable than the Tap-p62 complex.

We addressed this issue with a series of Tap deletion mutants in the context of a RevM10-Tap fusion that were used in a previous study to analyze Tap- and NXT1-dependent export of a reporter RNA (24). We reasoned that the NPC binding domain of Tap should be dispensable for binding p62 if NXT1 can bridge the interaction between these proteins. We first determined that RevM10-Tap (61–619), containing intact NXT1 and NPC binding domains, is functional in this assay

**FIG. 6. A Tap-binding mutant of NXT1 that fails to promote RNA export.** A, diagram of the reporter RNA that contains the RRE recognized by the RevM10-Tap fusion protein. The RNA transcript encodes the capsid protein p24 that is measured by enzyme-linked immunosorbent assay (24). B, Tap-dependent RNA export in the presence of FLAG-NXT1. RRE-gag/pol and RevM10-Tap plasmids were co-transfected in HeLa cells in conjunction with WT or mutant FLAG-NXT1 (R107A, K127A, E102N, and N48K/N50K), FLAG-NTF2, or empty FLAG vector as indicated. Levels of RNA export for each condition were measured as a function of p24 secretion. Each value represents the average of two data points obtained from independent transfection experiments. The error bars represent differences between duplicates.
Like the full-length Tap protein (1–619; Fig. 2B), the RevM10-Tap-(61–619) bound specifically to p62, and binding was stimulated by recombinant NXT1. In contrast, deleting 50 amino acids from the carboxyl terminus of RevM10-Tap-(61–569) abolished essentially all binding to p62. Significantly, including NXT1 in the assay failed to rescue RevM10-Tap-(61–569) binding to p62, despite the fact that this deletion mutant protein still binds to NXT1 (24). This indicates that NXT1 cannot bridge an interaction between Tap and p62 in the absence of the NPC binding domain of Tap. We also found that deletion of the NXT1-binding site from RevM10-Tap-(507–540) did not reduce its ability to bind p62 in the absence of NXT1. This indicates that NXT1 cannot bridge an interaction between Tap and p62 in the absence of the NPC binding domain of Tap. We also found that deletion of the NXT1-binding site from RevM10-Tap-(507–540) did not reduce its ability to bind p62 in the absence of NXT1. Finally, a RevM10-Tap mutant protein that lacks part of both the NXT1 and NPC binding domains (Δ507–570) displayed near background levels of binding to p62, both in the absence and presence of NXT1.

Our results indicate that Tap undergoes both NXT1-independent and NXT1-dependent interactions with p62 and that the NPC binding domain of Tap is necessary for both types of interactions. The carboxyl-terminal domain of Tap is predicted to perform a targeting function by initiating contact with the NPC. Subsequent interactions with the NPC, which are necessary for translocation of the export complex, are regulated by NXT1. This working model of NXT1 function is consistent with our recent finding that NXT1 is essential for Tap-dependent export of a reporter RNA (24).
compared with the WT protein, the NXT1 mutant N48K/N50K failed to stimulate Tap binding to p62 (Fig. 5C); this is despite the fact that the NXT1 mutant N48K/N50K binds to p62 at a level comparable with WT NXT1 (Fig. 5B). Thus, the NXT1-dependent binding of Tap to p62 requires a physical interaction between NXT1 and Tap. The R107A, K127A, and N48E NXT1 mutants stimulated Tap binding to p62 at levels that were near that of WT protein (Fig. 5C). The E102N and R107A mutants, which bind Tap near WT levels, were slightly reduced with regards to Tap binding to p62 (Fig. 5C) and in direct binding to p62 (Fig. 5B). The results of our analysis (Figs. 4 and 5) reveal that specific and efficient engagement of Tap with p62 requires protein-protein interactions between Tap and p62, Tap and NXT1, and NXT1 and p62. The cooperative binding of these three proteins, together with the fact that NXT1 stimulates Tap binding to nucleoporins in addition to p62, suggests a potential role for NXT1 in regulating Tap movement through the NPC.

**NXT1 Binding to Tap Is Necessary for RNA Export in Vivo**—Our binding analysis indicated that Tap undergoes both NXT1-independent and NXT1-dependent interactions with nucleoporin p62. Because NXT1 potently stimulates RNA export in the RevM10-Tap assay (24), we predicted that the NXT1-dependent interaction of Tap with p62, and potentially other nucleoporins, is a critical rate-limiting step of RNA export. To address this issue, we analyzed the activity of the NXT1 mutants in the RevM10-Tap assay that measures export of a RNA reporter (24). The readout for this assay is synthesis of p24, the HIV-1 capsid protein whose translation relies on nuclear export of the unspliced RNA. This requires an RNA-protein interaction between the RRE contained within the 3′ end of the RNA and Rev encoded by the RevM10-Tap fusion (Fig. 6A). Because the NES in Rev has been mutated, the nuclear export functionality in the system is conferred by Tap and its interaction with NXT1 (24). We transfected 293T cells with plasmids encoding RevM10-Tap, reporter RNA, WT, or mutant NXT1 proteins, as well as a secreted form of alkaline phosphatase to normalize transfection efficiency. As shown previously, co-transfection of WT NXT1 resulted in a significant stimulation of RNA export, as indicated by the increase in p24 production from <0.5 to 22.1 ng/ml (Fig. 6B). The functional interaction between NXT1 and Tap measured in this assay is highly specific because there was no enhancement of RNA export when NTF2 was substituted for NXT1 or when RevM10-Tap was omitted from the assay (Fig. 6B). The activities of the NXT1 mutants R107A, K127A, E102N, and N48E in Tap-dependent RNA export were comparable, albeit slightly less, to WT NXT1. In contrast, the NXT1 mutant N48K/N50K was severely impaired in its ability to stimulate Tap-dependent RNA export (Fig. 6B). Immunoblotting confirmed that the WT and mutant NXT1 proteins were expressed at similar levels, indicating that the results are not due to protein instability (data not shown). Taken together, our data suggest that NXT1-regulated interaction of Tap with nucleoporins, including p62, is a rate-limiting step in RNA export from the nucleus.

**NXT1 Regulates Binding of the Tap-CTE RNA Complex to p62**—The NXT1-dependent binding of Tap to p62 could reflect an interaction with the NPC that occurs during nuclear export of RNA. An alternative, although not a mutually exclusive possibility, is that NXT1 is a component of the recycling pathway for nuclear import of Tap. One major difference in these processes is that RNA will be bound to Tap during nuclear export but not during nuclear import. Thus, if NXT1 function were required during the RNA export process, it would be expected to stimulate binding of the Tap-RNA complex to nucleoporins such as p62. We addressed this possibility using a binding assay to quantitate both Tap and RNA binding within the same sample. The assay contained WT or transport-defective mutants of the CTE RNA, the cis-acting export signal present in type D retroviral transcripts (13, 18, 28). The predicted structure of the 183 nucleotide CTE includes two 12-nucleotide internal loops that are important for recognition of the RNA by Tap (14, 18).

We initially tested whether Tap can simultaneously bind NXT1 and CTE RNA using a co-IP assay. Because Tap uses different domains to bind RNA and NXT1 (17), we predicted that an NXT1-CTE RNA complex could be reconstituted in vitro. FLAG-NXT1 was incubated with anti-FLAG antibody immobilized to protein G beads in the presence of GST-Tap and 32P-CTE RNA (Fig. 7B). Under these conditions, 0.28 ± 0.06 fmol CTE RNA was recovered in the GST-Tap-FLAG-NXT1 complex. The protein-protein and protein-RNA interactions in this assay are specific because substituting GST for Tap in the reaction reduced CTE RNA binding to background levels (0.009 ± 0.001 fmol). Our results appear to be in conflict with those reporting that NXT1 and CTE binding to Tap is mutually exclusive (20). The reason for the discrepancy is unknown.

We tested whether Tap can mediate an interaction between CTE RNA and p62 using 35S-labeled Tap and 32P-labeled CTE RNA. We determined that the Tap-CTE RNA complex can bind to p62 and that the addition of recombinant NXT1 stimulates this interaction 6-fold (Fig. 7, C and D). NXT1 addition increases Tap binding to p62 without affecting the relative amount of RNA bound to Tap in this reaction. Thus, NXT1 does not appear to regulate the affinity of Tap for RNA cargo. We note, however, that addition of WT CTE RNA resulted in a modest stimulation of Tap binding to p62, both in the absence and presence of NXT1 (Fig. 7C). RNA binding was not observed when Tap was omitted from the assay, and only background binding was observed in the presence of Tap and the mutGAC RNA. The M2/M11 RNA displayed a higher level of binding than the mutGAC RNA, although even in the presence of NXT1, this totaled only ~36% of the WT CTE RNA.

NXT1-dependent binding of the Tap-RNA complex to p62 could be reconstituted using all recombinant proteins as well (Fig. 7E). This indicates that additional, lysate-derived factors are not required for assembly of the CTE RNA-Tap-NXT1-p62 complex.

**DISCUSSION**

In the present study, we have used several functional assays to characterize how the nuclear export factor, NXT1, regulates the activity of Tap during RNA export. Our results indicate that NXT1 regulates the interaction of Tap with the NPC by increasing the affinity of Tap for nucleoporins. Our analysis was focused on interactions involving p62, a nucleoporin that localizes to both the cytoplasmic and nucleoplasmic sides of the NPC where it provides binding sites for several different transport factors (4, 32). We found that Tap can bind to p62 by both NXT1-independent and NXT1-dependent mechanisms, which we suggest could reflect two alternate binding states of Tap.
during its translocation through the NPC. It is also conceivable that NXT1 targets Tap to a second class of binding sites on p62. Significantly, our observation that Tap, CTE RNA, and NXT1 display cooperative binding to p62 indicates that these interactions underlie the nuclear export pathway of the complex and not the recycling pathway for Tap.

The activity of NXT1 in Tap-dependent nuclear export has been examined previously in several model systems. The first indication that NXT1 and Tap might cooperate to mediate RNA export came from the finding that co-expression of these proteins can partially rescue growth in *S. cerevisiae* strains that harbor mutations in *MEX67* and *MTR2* (22). Mutations or deletions in *Mex67p* or *Mtr2p* induce nuclear accumulation of polyadenylated RNA, suggesting these are essential components of the mRNA export machinery (22, 25, 26, 35). The cross-species complementation result, together with co-IP data, suggested that NXT1 might be an essential subunit of Tap and that the Tap-NXT1 complex could be functionally similar to the Mex67p-Mtr2p complex (22). Interestingly, NXT1 is not encoded by the yeast genome and does not show significant sequence relatedness to *S. cerevisiae* Mtr2p (21). In contrast, human Tap and *S. cerevisiae* Mex67p are 21% identical at the amino acid level (expectation value $6 \times 10^{-10}$). Additional similarities between these complexes include the binding of NXT1 and Mtr2p to apparently analogous domains of Tap and Mex67p, and the involvement of NXT1 and Mtr2p in regulating Tap and Mex67p interactions with the NPC in vertebrates and yeast, respectively (20, 22, 35–37). Subsequent experiments from other laboratories seemed to disfavor the role of NXT1 as a subunit that is important for Tap function in higher eukaryotes. Tap proteins containing deletions that reduce, or possibly eliminate, binding to NXT1 were shown to be active for export of a CTE-containing reporter RNA in frog oocytes (20). Moreover, an NXT1-binding mutant of Tap transfected into quail cells could still stimulate export of a CTE at 50% the level of WT Tap (23). However, the activity of WT Tap in these studies might have been under-represented because NXT1 was not added, and NXT1 is clearly rate-limiting for Tap function in our assay. Nevertheless, the view that emerged from these latter studies was that NXT1 might play only a minor role in Tap-dependent RNA export.

In contrast, our analysis of Tap and NXT1 in mammalian cells has led us to conclude that the interaction between these proteins is critical for RNA export (24). Deletions within the NXT1 binding domain of Tap eliminate its ability to promote RNA export. Moreover, in the present study we have shown that mutations within the Tap binding domain of NXT1 (N48K/N50K) also eliminate the ability to promote RNA export. The Tap mutant Δ507–540 is particularly informative because it fails to bind NXT1, and it does not support RNA export (24). Tap-Δ507–540 displays a basal level of binding to p62 but does not display NXT1-stimulated binding to p62 (summarized in Table 1). As discussed below, these and other results indicate that Tap-dependent RNA export requires productive binding between Tap and NXT1, the function of which is to regulate Tap interactions with the NPC. The interaction between Tap and NXT1 appears to be important for export of cellular mRNA as well. Co-injections of Tap and NXT1 into the oocyte nucleus can enhance (~3-fold) export of adenovirus mRNA (38). Nuclear export of certain other RNAs, however, did not appear to require the Tap-NXT1 heterodimer (38). One interpretation of these results is that the requirement for NXT1 on Tap export pathways may depend on the nature of the particular RNA cargo.

Whereas NXT1 and Tap have a predominantly nuclear distribution, both proteins undergo nucleocytoplasmic shuttling during the course of RNA export and recycling back into the nucleus (18, 19, 21). A subset of both NXT1 and Tap is detected at the NPC following mild extraction with detergents (19–22). We showed by IF that this reflects, at least in part, the association of Tap and NXT1 with the cytoplasmic side of the NPC. Whether the subset of NXT1 and Tap that is bound to the cytoplasmic side of the NPC represents an outgoing RNA-containing complex in the export pathway or an incoming RNA-free complex in the import pathway is presently unknown. By using immuno-EM, we found that NXT1 is associated with cytoplasmic and the nuclear side of the NPC in HeLa cells, and a similar observation has been reported for Tap in the frog oocyte (20). The average distribution of NXT1, which is ~40 nm from the central plane of the NPC, places it proximal to the central gated channel of the NPC near the location of the p62 complex (32). Because NXT1 shuttles between the nucleus and cytoplasm, this average distribution could be interpreted as evidence that a rate-limiting transport event occurs at this site on the NPC.

Expressed sequence tag CDNA analysis suggests that humans contain at least five isoforms of Tap and three isoforms of NXT1 (39). It is reasonable to suggest, therefore, that distinct complexes might be assembled from the different isoforms of Tap and NXT1. These complexes could, in turn, be responsible for transporting different classes of cellular RNA. It has been shown recently that depletion of a particular isoform of Tap in Caenorhabditis elegans by RNA interference leads to partial defects in polyadenylated RNA export (40). This result provides good support for the current working hypothesis in the export field that Tap plays a role in cellular RNA export. Defining the specific transcripts and cis-acting export signals within cellular RNAs that may be recognized by the Tap-NXT1 complex remains a highly important issue for study.

We addressed how NXT1 may influence Tap interactions with the NPC in a series of binding experiments, using p62 as the model nucleoporin. We found that Tap could bind specifically to p62 and that binding was stimulated up to 6-fold by the addition of recombinant NXT1. The presence of NXT1 in the reaction increased both the affinity and total level of Tap binding to p62. These results could be explained by NXT1 acting as an adapter that bridges the interaction between Tap and p62, because NXT1 can bind to both of these proteins. Alternatively, NXT1 could modify the structure of Tap in a manner that increases its affinity for p62. We found that a Tap mutant (61–569), which binds to NXT1, is unable to bind p62 in the absence or presence of NXT1; this same mutant does not support RNA export (24). This provides evidence that NXT1 does not simply bridge the interaction between Tap and p62. Rather, we propose that NXT1 binding to Tap induces a structural change in the carboxyl-terminal domain of Tap, which increases its affinity for nucleoporins. The NXT1 binding domain in Tap is, in fact, adjacent to the carboxyl-terminal domain that directly contacts nucleoporins such as p62. Because NXT1 itself binds to p62, it is certainly possible that Tap, NXT1, and p62 may undergo simultaneous interactions during nuclear export. Although we have not mapped the exact sites on p62 that are contacted by Tap and NXT1, our analysis of deletion derivatives of the nucleoporin p58 indicates the export complex interactions occur within the amino- and carboxyl-terminal domains that contain FXFG motifs and not within middle domain that lacks these motifs. Our interpretation is consistent with previous results showing that Tap binds to the FXFG-containing domains of the nucleoporin CAN/Nup214 and that the Mex67p/Mtr2p heterodimer binds to several different FG-containing nucleoporins (20, 22, 36).

We were able to demonstrate the assembly and NXT1-de-
dependent binding of the Tap-CTE RNA export complex to the nucleoporin p62. To our knowledge, this is the first reconstitution of RNA-protein complex binding to a component of the NPC in vitro. Tap binding to the CTE RNA in our assay system is highly specific because Tap can discriminate between a transport functional and nonfunctional CTE within the RNA. A unique and novel aspect of our approach was to monitor radiolabeled Tap and CTE RNA in the same sample, which enabled us to show directly that NXT1 promotes the binding of the Tap-CTE RNA complex to p62. We also observed that NXT1 and the CTE RNA could each promote Tap binding to p62, an indication that export complex interactions with the NPC can be influenced by a receptor subunit (NXT1) that does not directly contact the RNA, as well as by the associated cargo (RNA).

In our initial characterization of NXT1, we showed that NXT1 binds to RanGTP and that it stimulates nuclear export of NES-containing proteins on a pathway that relies on Crm1 as the export receptor (21). More recently, we found that NXT1 cooperates with RanBP1 to promote the release of an export complex containing Crm1, RanGTP, and NES protein from the cytoplasmic side of the NPC (27). Our data addressing the Crm1 pathway suggested that NXT1 co-assembles with the Crm1-NES-RanGTP export complex in the nucleoplasm, and promotes movement of the complex to a site on the NPC where disassembly and release occur (27). It is reasonable to propose that the NXT1-dependent binding of the Tap-CTE RNA complex to nucleoporins as described in the present study may be functionally related to NXT1-dependent movement of the Crm1-NES-RanGTP complex to and release from nucleoporins within the NPC. In the two export pathways, Tap and Crm1 appear to use NXT1 to help specify targeting to particular sites within the NPC. Whether NXT1 is involved in disassembly and release of the Tap-CTE RNA complex and whether Ran is involved in Tap-dependent RNA export are important questions for future study. Although experiments performed in yeast support a role for Ran in multiple RNA export pathways (41), recent experiments performed in the oocyte suggest that nuclear export of spliced RNA can occur independently of Ran (42).

In summary, we have identified a specific function for NXT1 on the Tap-dependent RNA export pathway. NXT1 binding to Tap increases the affinity of the Tap-CTE RNA complex for nucleoporins within the NPC. Our data support the view that transport through the NPC may be governed by the affinity of cargo-bound receptors for spatially distinct nucleoporins (43). The NXT1-dependent association of the Tap-CTE RNA complex with the nucleoporin p62, in particular, is likely to reflect a transient intermediate that occurs during translocation of the ribonucleoprotein complex through the NPC.

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RNA Export Mediated by Tap Involves NXT1-dependent Interactions with the Nuclear Pore Complex

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