MOLECULAR AND CELLULAR BIOLOGY, Dec. 1999, p. 8616–8624 Vol. 19, No. 12
0270-7306/99/$04.00+0

Identification of an NTF2-Related Factor That Binds Ran-GTP and Regulates Nuclear Protein Export

BEN E. BLACK,[1,2,3] LYNE LÉVESQUE,[1,2] JAMES M. HOLASKA,[1] TODD C. WOOD,[2] AND BRYCE M. PASCHAL[1,2,3]*

Center for Cell Signaling,[1] Department of Biochemistry and Molecular Genetics,[2] and Cell and Molecular Biology Program,[3] University of Virginia Health Sciences Center, Charlottesville, Virginia 22908

Received 14 July 1999/Returned for modification 24 August 1999/Accepted 3 September 1999

Active transport of macromolecules between the nucleus and cytoplasm requires signals for import and export and their recognition by shuttling receptors. Each class of macromolecule is thought to have a distinct receptor that mediates the transport reaction. Assembly and disassembly reactions of receptor-substrate complexes are coordinated by Ran, a GTP-binding protein whose nucleotide state is regulated catalytically by effector proteins. Ran function is modulated in a noncatalytic fashion by NTF2, a protein that mediates nuclear import of Ran-GDP. Here we characterize a novel component of the Ran system that is 26% identical to NTF2, which based on its function we refer to as NTF2-related export protein 1 (NXT1). In contrast to NTF2, NXT1 preferentially binds Ran-GTP, and it colocalizes with the nuclear pore complex (NPC) in mammalian cells. These properties, together with the fact that NXT1 shuttles between the nucleus and the cytoplasm, suggest an active role in nuclear transport. Indeed, NXT1 stimulates nuclear protein export of the NES-containing protein PKI in vitro. The export function of NXT1 is blocked by the addition of leptomycin B, a compound that selectively inhibits the NES receptor Crm1. Thus, NXT1 regulates the Crm1-dependent export pathway through its direct interaction with Ran-GTP.

Protein and RNA transport between the nucleus and cytoplasm occurs through nuclear pore complexes (NPCs), elaborate proteinaceous channels that span the double-membrane system of the nuclear envelope (8, 18, 26, 27). Transport through the NPC requires soluble receptors that recognize a nuclear localization signal (NLS) or a nuclear export signal (NES) within a protein destined for import or export, respectively. Upon binding to NLS or NES cargo, receptors mediate transport of the receptor-cargo complex through the central gated channel of the NPC in a poorly understood translocation reaction. The receptor-cargo complex is subsequently disassembled, and the receptor is recycled to the original compartment for additional rounds of transport.

In addition to NLS and NES receptors, nuclear import and export pathways require the direct participation of Ran, a small GTP-binding protein of the Ras superfamily (8, 27). Like other Ras-related GTPases, Ran adopts different conformations in its GDP- and GTP-bound states (49). The conformation of Ran-GDP facilitates an interaction with RCC1 to catalyze nucleotide exchange, whereas the conformation of Ran-GTP facilitates an interaction with the GTPase-activating protein RanGAP to stimulate nucleotide hydrolysis. Because RCC1 is nuclear and RanGAP is cytoplasmic, a steep gradient of Ran-GTP/Ran-GDP is predicted to exist across the nuclear envelope (11, 39). The best-understood functions of Ran in nuclear transport are assembly and disassembly reactions of transport complexes. For example, nuclear Ran-GTP assembles into a complex with the export receptor Crm1 and NES cargo; upon reaching the cytoplasm, disassembly of the complex is triggered by RanGAP-stimulated GTP hydrolysis (10). The export of mRNA from the nucleus is also thought to be receptor mediated and dependent on Ran-GTP, but the specific contributions of transport factors to this pathway are much less clear than for protein export. *GLE1* is clearly involved in mRNA export in *S. cerevisiae* (30), and recent characterization of its human homologue indicates this function is conserved in higher eukaryotes (50). Analysis of *MEX67* in *S. cerevisiae* and its apparent mammalian orthologue TAP has revealed a role for these proteins in mRNA export as well (47). TAP was functionally characterized as an mRNA export factor based on its ability to stimulate nuclear export of mRNA that contains the constitutive transport element found in simple retroviruses (12), and it may mediate host mRNA export as well (3, 21). While these observations suggest that RNA export involves multiple soluble proteins, delineating the machinery directly responsible for nuclear translocation of RNA has proven elusive. What is clear is that these pathways all converge on the NPC and are predicted to depend on the GTP-bound form of Ran (47).

Ran-GDP targeting to the nucleus is mediated by NTF2 (38, 43), a highly conserved protein originally identified by its ability to stimulate nuclear import in digitonin-permeabilized cells (29, 36). NTF2 also binds directly to NPC proteins located near the central gated channel (13, 17), a property consistent with mediating nuclear translocation of Ran. *S. cerevisiae NTF2* is required for viability and it shows genetic interactions with *GSP1*, the gene encoding the yeast homologue of Ran (7, 35, 52). NTF2 binds Ran-GDP (31, 34) but fails to bind Ran-GTP due to nucleotide-dependent conformations of Ran that alter the site of interaction (49). Moreover, we have obtained direct evidence that NTF2 regulates the distribution of Ran in living cells by showing that its nuclear import is blocked by monoclonal antibodies to NTF2 (45). Thus, the properties of NTF2 revealed by transport assays, in vitro binding studies, and conditional mutants in *S. cerevisiae* all support the view that it plays an important role in nuclear protein import. In addition, nuclear microinjection of a high concentration of NTF2 blocks protein export in tissue culture cells (48). Determination of

* Corresponding author. Mailing address: 7161 Hospital West, Box 577 Health Science Center, University of Virginia, Charlottesville, VA 22908. Phone: (804) 243-6521. Fax: (804) 924-1236. E-mail: paschal@virginia.edu.

whether this reflects a primary role in nuclear protein export requires further analysis.

Here we identify a novel transport factor, structurally related to NTF2, that binds specifically to Ran-GTP. NXT1 shuttles between the nucleus and cytoplasm and accumulates at the NPC. Significantly, NXT1 stimulates Crm1-dependent nuclear protein export of protein kinase inhibitor (PKI) in a permeabilized cell assay (16). These properties indicate that NXT1 regulates nuclear export through its interaction with Ran-GTP.

MATERIALS AND METHODS

Identification of NXT1 ORFs and phylogenetic analysis. Plasmids containing the full-length open reading frame (ORF) of NXT1 from human, mouse, and fly cells were obtained from the Integrated Molecular Analysis of Genomes and Their Expression (I.M.A.G.E.) consortium. The worm NXT1 clone was obtained from the National Institute of Genetics, Japan (kindly provided by Yuji Kohara). Each cDNA clone was sequenced to determine NXT1 sequences. The phylogenetic tree was constructed by the Fitch-Margoliash method (9). All sequences were aligned by using CLUSTAL W version 1.6. Phylogenies were constructed by using protdist, fitch, neighbor, and protpars programs of the PHYLIP package (version 3.5c; distributed by the author, J. Felsenstein), using the default parameters.

Yeast methods. Experiments using *S. cerevisiae* were performed by standard methods (14). The E40N mutation was made with the QuikChange site-directed mutagenesis system (Stratagene, La Jolla, Calif.) and confirmed by sequencing. 5-Fluoro-orotic acid was used at 1 mg/ml. The NXT1 overexpression plasmid was constructed by cloning the ORF from mouse NXT1 into an expression vector (pMSS32) containing the *ADH1* promoter. Individual colonies were restreaked on the indicated media and incubated for 2 days at 30°C.

Purification of recombinant NXT1. The ORF of mouse NXT1 was cloned into a modified version of pET23b (Novagen, Inc., Madison, Wis.) that lacks the T7 epitope tag. NXT1 protein was expressed in *Escherichia coli*(BL21) and solubilized from the inclusion body fraction with 8 M urea in TNE buffer (20 mM Tris [pH 8.0], 50 mM NaCl, 2 mM EDTA, 2 mM dithiothreitol). The solubilized fraction was fractionated by gel filtration chromatography (S300; Pharmacia) in 8 M urea–TNE buffer. Following exhaustive dialysis against TNE buffer, the protein was purified by chromatography on DEAE-Sepharose, using a linear salt gradient. The NXT1 protein, which eluted with ~240 mM NaCl, was dialyzed against TNE, dispensed into single-use aliquots (concentrations, 0.5 mg/ml), and frozen in liquid N_2. Since a single freeze-thaw cycle appears to induce aggregation of purified NXT1 protein, we routinely clarify the protein (40,000 × *g* for 30 min).

Solid-phase binding and GAP assays. For the binding assays, recombinant NXT1 and NTF2 were absorbed to 96-well plates in transport buffer (36) overnight at 4°C. The wells were then blocked with 30 mg of bovine serum albumin (BSA) per ml in transport buffer for at least 6 h. Ran (preloaded with radiolabeled GDP or GTP) was added to each well in 0.5× transport buffer containing 5 mg of BSA per ml. After incubation overnight at 4°C, the wells were washed three times, and bound protein was eluted with 5% sodium dodecyl sulfate (SDS) and analyzed by scintillation counting or by thin-layer chromatography. Binding to wells containing BSA alone (background) was subtracted from NXT1 and NTF2 wells. All binding assays were performed in duplicate or triplicate, and values are presented as mean ± standard deviation (SD). To determine the affinity of NXT1 for Ran-GTP, binding reactions were performed with increasing concentrations of Ran preloaded with [γ-^{32}P]GTP. The bound counts were measured by scintillation counting, and the data were fit to a curve with the least squares method, using the program SigmaPlot version 3. The K_D of the NXT1-Ran interaction was measured to be 8.5 nM.

GAP assays were performed with Ran (0.4 μM) preloaded with [α-^{32}P]GTP and recombinant RanGAP. Reactions were performed at 30°C in transport buffer alone or in the presence of NXT1 (7 μM) or RanBP1 (7 μM). The relative amount of GDP- and GTP-bound Ran was measured by thin-layer chromatography and analysis on a phosphorimager. All GAP assays were performed in duplicate, and values are presented as mean ± SD.

Heterokaryon analysis and protein export assay. pFlag-NXT1 was constructed by cloning the mouse NXT1 ORF into pcDNA-Flag, and the plasmid was transfected into HeLa S3 cells by electroporation. Stable transfectants (donor cell line) were selected in G418-containing Dulbecco modified Eagle medium containing 10% newborn calf serum. The green fluorescent protein (GFP)-NLS-expressing cell line (acceptor cell line), generated in a similar manner, stably expresses a nondiffusible GFP fusion protein including streptavidin and the NLS from simian virus 40 large T antigen. The donor and acceptor cell lines were coplated onto glass coverslips and incubated overnight. Cells were treated with cycloheximide (100 μg/ml) for 30 min prior to the addition of 50% polyethylene glycol (28). Following fusion, heterokaryons were washed four times in medium and incubated for an additional 2 h in the presence of cycloheximide. Cells were then fixed and processed for immunocytochemistry as described below.

Export reactions were performed as described elsewhere (16). Cytosol with or without NXT1 was pretreated with leptomycin B (LMB; 500 nM) for 1 h at 4°C, and the samples were directly added to the appropriate export reactions.

Immunofluorescence. Cells were washed in phosphate-buffered saline (PBS; 137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.76 mM KH_2PO_4 [pH 7.4]) and fixed in 4% formaldehyde in PBS for 10 min at room temperature. Coverslips were then washed three times in PBS and permeabilized with 0.2% Triton X-100 in PBS (20 mM Tris, 154 mM NaCl [pH 7.4]) for 30 min. Coverslips were washed three times in PBS-T (PBS with 0.1% Tween 20) and incubated in blocking solution (2% BSA, 2% fetal bovine serum, and 0.1% Tween 20 in PBS) for 1 h at room temperature.

Coverslips were incubated with primary antibody and diluted with blocking solution overnight at 4°C. The coverslips were washed three times in PBS-T and incubated in secondary antibody diluted with blocking solution for 1 h at room temperature. NXT1 was detected with the anti-FLAG monoclonal antibody M2 (1:5,000; Sigma). The monoclonal antibody to the NPC (RL1; kindly provided by Larry Gerace) (44) was used at a dilution of 1:100, and the polyclonal antibody to Ran was used at 20 μg/ml (45). Goat anti-mouse Cy3-conjugated secondary antibody and goat anti-rabbit fluorescein isothiocyanate-conjugated secondary antibody were obtained from Jackson Laboratories. An immunoglobulin M μ-chain-specific goat anti-mouse rhodamine-conjugated antibody (Pierce) was used for detection of RL1 antibodies. Cells were then washed three times in PBS-T and mounted with Vectashield mounting medium (Vector Laboratories Inc.). Digital images were captured by a charge-coupled device camera (Hamamatsu ORCA) mounted on a Nikon Microphot-SA microscope, using Openlab software 2.0.6, and processed as color images by Photoshop 5.0 and Freehand 8.0.

Nucleotide sequence accession numbers. The GenBank accession numbers for the NXT1 sequences are AF156957 (human), AF156958 (mouse), AF156959 (fly), and AF156960 (worm).

RESULTS

Identification of an NTF2-related factor. We searched the *Caenorhabditis elegans* genome database to identify proteins with significant sequence similarity to the yeast protein NTF2 (5). The results of our BLAST searches revealed two ORFs that are 32 and 43% identical to the NTF2 protein from *S. cerevisiae*. Since known plant, animal, and fungal NTF2 proteins share ~44% identity, the more closely related *C. elegans* protein likely corresponds to NTF2 and functions in nuclear targeting of Ran-GDP (38, 43). The sequence divergence of the more distantly related *C. elegans* protein prompted us to hypothesize that the nuclear transport machinery of worms, and perhaps other higher eukaryotes, requires a distinct NTF2-related protein. Based on the functional properties described in this study, we named this protein NTF2-related export protein 1 (NXT1). We looked for orthologues of NXT1 by searching EST (expressed sequence tag) databases with the predicted protein sequence of *C. elegans* NXT1, using the TFASTX program (37). EST clones with significant similarity to *C. elegans* NXT1 were sequenced to determine the complete primary structures of human, mouse, and fly NXT1 (Fig. 1A). To infer the evolutionary history of these proteins, the multiple sequence alignment of NTF2 and NXT1 proteins was used to construct a phylogenetic tree (9). The phylogeny clearly shows two paralogous branches, one consisting of animal NTF2 sequences and the other consisting of animal NXT1 sequences (Fig. 1B). No orthologues of NXT1 were detected in the *S. cerevisiae* genome or in any fungal or plant EST projects. We conclude that NXT1 is a paralogue of NTF2 that is unique to animals.

NXT1 and NTF2 are related but functionally distinct. To visually assess the extent of sequence similarity between NTF2 and NXT1, we constructed a model of mouse NXT1 based on the atomic structure of rat NTF2 (4) (Fig. 2A). Similarity between the two structures includes seven of nine hydrophobic residues that in NTF2 form a binding pocket for Phe72 of Ran (46). The structural congruence in the Ran-binding site implies that NXT1 may also interact with Ran; however, the functionally important and conserved Glu42 in rat NTF2 is replaced with Asn48 in mouse NXT1. Mutation of Glu42 to lysine abol-

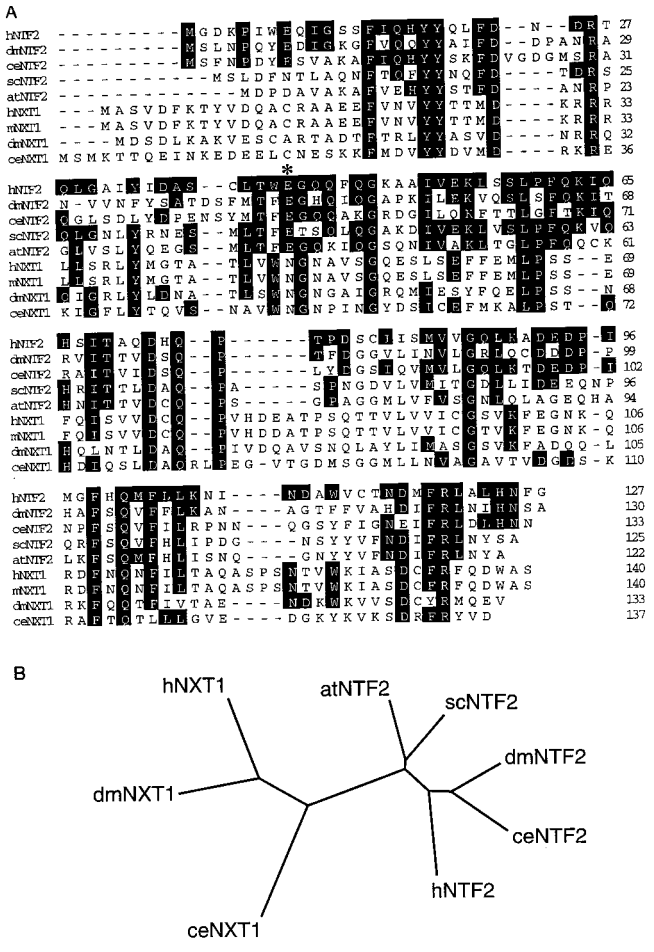

FIG. 1. Comparison of NTF2 and NXT1 proteins from diverse species. (A) The primary structure of NTF2 proteins from human (hNTF2), fly (dmNTF2), worm (ceNTF2), yeast (scNTF2), and *Arabidopsis* (atNTF2) were aligned with NXT1 proteins from human (hNXT1), mouse (mNXT1), fly (dmNXT1), and worm (ceNXT1). Human NXT1 and NTF2 are 26% identical over 118 amino acids. The invariant glutamate in NTF2 (Glu42 in hNTF2) that is required for binding Ran-GDP is indicated (*). Notably, NXT1 contains an invariant asparagine at this position (Asn48 in hNXT1). Mouse NXT1 also shows 30% identity to amino acids 14 to 135 of RasGAP SH3-binding protein (33) (accession no. AF051311), though the biological significance of this relationship is unknown. (B) Phylogeny of NTF2/NXT1 generated from the sequence alignment (9). Mouse NXT1 was omitted from the phylogeny because it is 99% identical to its human orthologue. Phylogenies of the same sequences using the neighbor-joining and maximum parsimony methods yielded identical topologies (data not shown). Mtr2p was not included in the phylogeny because its sequence is not significantly similar to that of NXT1, despite the fact that the proteins have functional similarities (21). In a FASTA search of the SwissProt database, the alignment of yeast Mtr2p and mouse NXT1 protein sequences has an expectation value of 340. In contrast, the alignment of mouse NXT1 and yeast NTF2 has an expectation value of 10^{-4}. NXT1 is also not related to the Ran-GTP-binding protein Mog1p, a recently described nuclear import factor that interacts genetically with *NTF2* in the budding yeast *S. cerevisiae* (32).

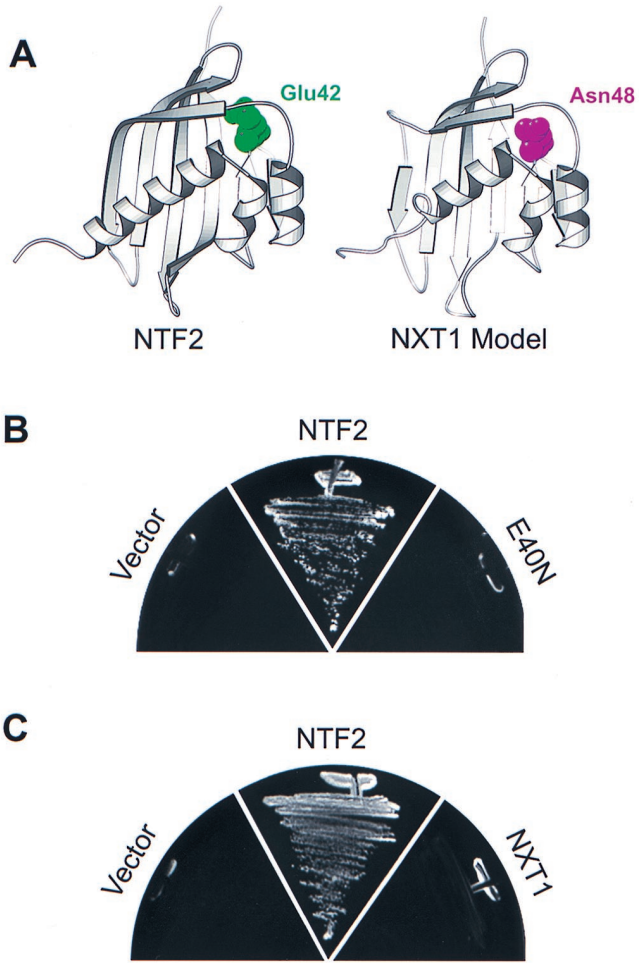

FIG. 2. NXT1 is functionally distinct from NTF2. (A) Ribbon diagram of the NTF2 monomer from the crystal structure (4) (Protein Data Bank no. 1oun, chain A) compared to the structure of mouse NXT1 generated with the Modeller program. The invariant glutamate in the Ran-binding domain of NTF2 is replaced with an invariant asparagine in NXT1. (B) BPY4(YCplac33-*NTF2*) was transformed with pRS315 (vector), pRS315-*NTF2* (NTF2), or pRS315-*ntf2* E40N (E40N). Individual colonies were restreaked on 5-fluoro-orotic acid-containing medium. The E40N mutation was lethal, as there was no growth in this strain upon counterselection of the wild-type copy. (C) BPY4(pRS315-*MET3-NTF2*) was transformed with pMSS32 (vector), YEp24-*NTF2* (NTF2), or pMSS32-NXT1 (NXT1). Individual colonies were restreaked on medium containing 5 mM methionine. Upon repression of *NTF2* expression, strains containing a plasmid copy of *NTF2* were viable, but those containing vector alone or a plasmid copy of NXT1 failed to grow.

ishes the nuclear import function of rat NTF2 in vitro and yeast *NTF2* in vivo by eliminating its interaction with Ran (6).

As a first step in evaluating the functional relatedness of NTF2 and NXT1 in vivo, we mutated the corresponding glutamate residue in budding yeast *NTF2* (Glu40) to asparagine and tested whether the resulting protein (E40N) could substitute for wild-type *NTF2* in a plasmid shuffle experiment (Fig. 2B). Wild-type *NTF2* expressed from a plasmid complemented growth in the *ntf2* deletion strain BPY4 (middle segment). In contrast, *ntf2* containing the E40N mutation failed to complement growth in the same strain (right segment). We next tested whether mouse NXT1 overexpression could complement growth in the *ntf2* deletion strain. Yeast cells in which *NTF2* expression is repressed failed to grow when NXT1 was overexpressed (right segment), demonstrating that NXT1 cannot functionally substitute for *NTF2* in budding yeast. Thus, although the predicted structural similarity of NXT1 and NTF2 suggests a role for NXT1 in nuclear transport, our mutational and complementation data show that these proteins have distinct functions in the cell.

NXT1 binds specifically to Ran-GTP. The interaction of Ran with NTF2 is mediated by residues within the flexible switch II region of the GTPase, a domain whose structure depends on whether Ran contains GDP or GTP (46). Since the switch II region adopts a more open conformation when Ran is in the GTP-bound form (49), we reasoned that this form of

Ran might interact with NXT1. We tested this hypothesis in solid-phase binding assays with recombinant NXT1 and NTF2 immobilized in microtiter wells. Recombinant Ran preloaded with radiolabeled [^{3}H]GDP or [γ-^{32}P]GTP was added to the wells, and the interaction was quantitated by measuring the number of counts per minute in the bound fraction. GDP-Ran binding to NTF2 wells was 6.5-fold greater than that observed in NXT1 wells (Fig. 3A). In contrast, GTP-Ran binding to NXT1 wells was 5.7-fold greater than that observed in NTF2 wells (Fig. 3B). We performed related experiments by incubating NTF2 and NXT1 wells with Ran preloaded with a mixture of [α-^{32}P]GDP and [α-^{32}P]GTP. The bound fractions were subjected to thin-layer chromatography, and the ratio of radiolabeled GDP and GTP forms of Ran were compared to the total amount of Ran bound in each well. NTF2 preferentially bound GDP-Ran, while NXT1 preferentially bound GTP-Ran (Fig. 3C and D). To further characterize the interaction between NXT1 and Ran, solid-phase binding assays were performed with Ran preloaded with [γ-^{32}P]GTP over a range of Ran concentrations (Fig. 3E). This analysis revealed that the affinity of NXT1 for Ran-GTP is 8.5 nM. The affinity is slightly less than the low-nanomolar affinity of RanBP1 for Ran-GTP (23). We note that the affinity of NXT1 for Ran-GTP could be modulated by other proteins in vivo, given that NXT1 probably interacts directly with soluble transport factors and the NPC (21) (see below).

NXT1 is not a RanGAP cofactor. One well-characterized function of known Ran-GTP-binding proteins is an ability to promote guanine nucleotide hydrolysis. RanGAP directly catalyzes GTP hydrolysis by Ran (1), while Ran-GTP-binding proteins RanBP1 and RanBP2 can serve as coactivators of this reaction (2, 25, 40). By modulating the GTPase cycle, these Ran-binding proteins are key regulators of Ran-dependent nuclear import and export. The binding specificity of NXT1 for Ran-GTP led us to examine whether this interaction modulates guanine nucleotide hydrolysis by Ran. We found that NXT1 alone does not activate the GTPase activity of Ran (data not shown). To determine if NXT1 regulates RanGAP-mediated turnover of Ran-GTP, we performed GAP assays (Fig. 4A) in the absence or presence of a large excess (7 μM) of NXT1 protein. We found that NXT1 neither inhibits nor stimulates RanGAP activity. To confirm that our RanGAP assay conditions are sensitive to coactivators, we performed RanGAP reactions in the presence RanBP1 and measured conversion of GTP-Ran to GDP-Ran as a function of time. Though the reactions were performed in duplicate for purposes of quantitation, single time points from the thin-layer chromatography are shown for illustration (Fig. 4B). After a 4-min incubation, the reactions supplemented with buffer or NXT1 still contained 40.6% $\pm$ 1.7% or 40.9% $\pm$ 0.4% of GTP-Ran, respectively. At the same time point, the reaction supplemented with RanBP1 contained only 21.2% $\pm$ 0.9% of the GTP-Ran. Thus, under conditions where RanGAP-mediated hydrolysis can be enhanced by RanBP1, NXT1 has no apparent stimulatory effect. This indicates that unlike other Ran-GTP-binding proteins, the function of NXT1 binding to Ran is not to regulate its GTPase cycle. We reasoned, therefore, that NXT1 might regulate Ran function in a stoichiometric rather than catalytic manner. For example, NXT1 could regulate the localization of Ran-GTP in a manner analogous to nuclear localization of Ran-GDP by NTF2 (38, 43, 45).

NXT1 localizes to the NPC. We examined the subcellular distribution of NXT1 by immunofluorescence microscopy in a HeLa cell line stably transfected with Flag epitope-tagged mouse NXT1. NXT1 localizes exclusively to the nucleus in interphase cells, similar to Ran (Fig. 5A) and NTF2 (45).

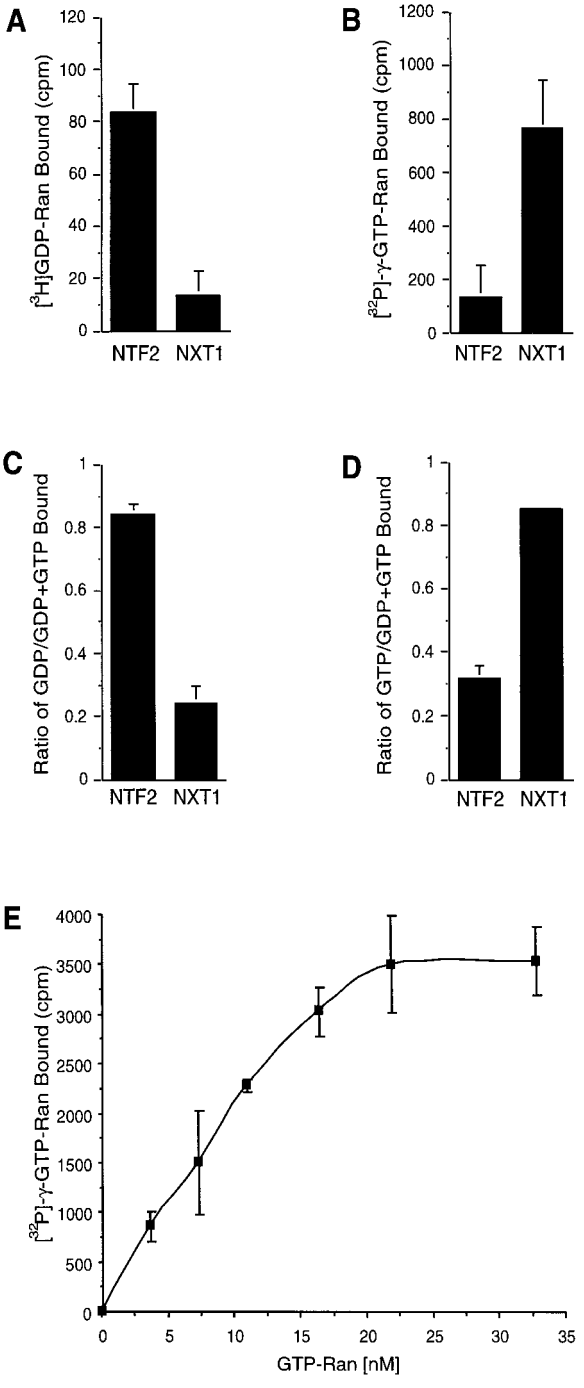

FIG. 3. NTF2 and NXT1 interact with different nucleotide-bound forms of Ran. Recombinant NTF2 and NXT1 were immobilized in microtiter wells, to which Ran preloaded with [^{3}H]GDP-Ran (A) or [γ-^{32}P]GTP-Ran (B) was added. NTF2 preferentially bound GDP-Ran (A), whereas NXT1 preferentially bound GTP-Ran (B). (C and D) Binding assays were also carried out with mixtures of [α-^{32}P]GDP-Ran and [α-^{32}P]GTP-Ran. When presented with mixed nucleotide forms of Ran, NTF2 and NXT1 specifically bound to GDP-Ran and GTP-Ran, respectively. (E) The affinity of Ran binding was measured by performing binding reactions with increasing concentrations of Ran preloaded with [γ-^{32}P]GTP.

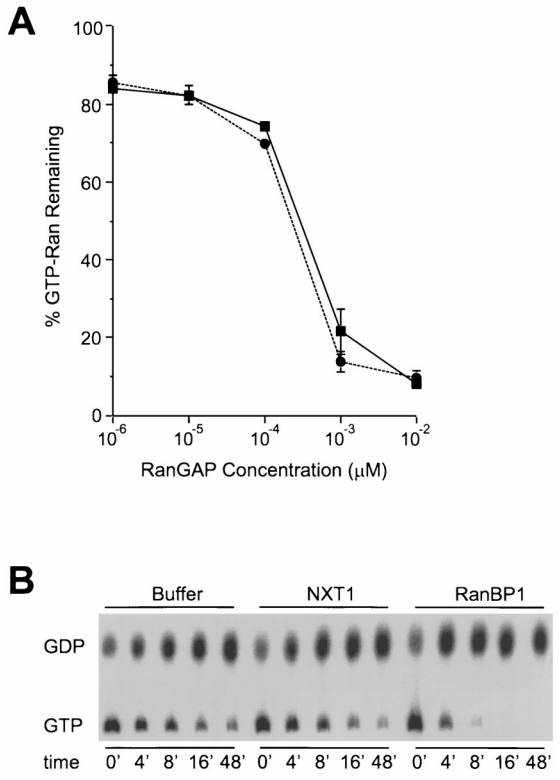

FIG. 4. NXT1 does not affect RanGAP-stimulated GTP hydrolysis of Ran. (A) Recombinant Ran preloaded with [α-^{32}P]GTP was incubated with recombinant RanGAP for 10 min with (●) or without (■) NXT1. The reactions were terminated by the addition of SDS and analyzed by thin-layer chromatography. (B) Similar GAP assays were carried out with Ran preloaded with [α-^{32}P]GTP and incubated with RanGAP (1 nM) in the presence of buffer alone, NXT1 (7 μM), or RanBP1 (7 μM). Samples were taken at 0, 4, 8, 16, and 48 min and analyzed by thin-layer chromatography.

NXT1 localization was also evident at the nuclear envelope, a distribution that was visualized clearly when cells were extracted with digitonin to release soluble proteins including Ran (Fig. 5A). The discontinuous, punctate distribution of NXT1 at the nuclear envelope is consistent with a localization at the NPC. To directly establish the NPC localization, HeLa cells were double labeled with anti-Flag (red) and a pan-nucleoporin antibody (44) (RL1; green), and the resulting images were merged to reveal the extent to which the distributions overlap (Fig. 5B). The bright yellow punctate signal at the nuclear envelope in the merged image demonstrated that NXT1 localizes to NPCs.

NXT1 is a shuttling protein. Our observations that NXT1 binds Ran-GTP and localizes to the NPC strongly suggested that its function is related to nuclear transport. Since most nuclear transport factors undergo continuous exchange between the nucleus and cytoplasm, we performed heterokaryon cell fusion experiments to determine if NXT1 undergoes nucleocytoplasmic shuttling. These experiments involved fusing the cell line stably expressing Flag-NXT1 (donor cells) with a cell line stably expressing a nucleus-localized GFP reporter (acceptor cells). The acceptor cell line used in this analysis was generated by transfecting a plasmid encoding GFP-streptavidin fused to the NLS of simian virus 40 large T antigen. Since the encoded polypeptide is too large to diffuse through the NPC and shows a constitutively nuclear distribution, it distinguishes acceptor cell nuclei from donor cell nuclei in the fluorescence microscope. The two cell lines (donor and acceptor) were fused in the presence of polyethylene glycol, and the distribution of NXT1 and GFP-NLS was examined by fluorescence microscopy. NXT1 localized to all four nuclei of a heterokaryon containing one donor cell nuclei and three acceptor cell nuclei (Fig. 6). In contrast, the non-shuttling GFP-NLS remained within the three acceptor cell nuclei of the heterokaryon. This result shows that NXT1 was exported from the donor cell nucleus and subsequently imported into the acceptor cell nuclei. The shuttling behavior of NXT1 was observed in multiple heterokaryons from three separate experiments.

NXT1 stimulates protein export from the nucleus. To examine directly whether NXT1 functions in nuclear transport, we tested the recombinant protein for its ability to stimulate nuclear import and export in digitonin-permeabilized cell assays. We found that NXT1 had no effect on nuclear import of NLS-containing reporter proteins under several conditions examined. To determine if NXT1 is an export factor, we tested its ability to stimulate nuclear export of a complex of biotinylated PKI and fluorescently labeled streptavidin (16). Addition of a saturating concentration of HeLa cell cytosol (2 mg/ml) to the assay promotes a maximal level of nuclear export of the PKI complex; including a subsaturating concentration of cytosol (0.5 mg/ml) induces only a low level of export (Fig. 7A). Addition of recombinant NXT1 to a reaction containing the subsaturating concentration of cytosol resulted in a level of export comparable to the maximal level obtained with 2 mg of cytosol per ml. Maximal stimulation of PKI export was observed with 40 μg of NXT1 protein per ml (Fig. 7B). We note that a role for NXT1 in nuclear export is consistent with our finding that it binds directly to Ran-GTP. The GTP form of Ran is required for export (39), the function of which is to promote the assembly of transport complexes that contain the export receptors such as Crm1 (10, 24). To determine if NXT1 stimulates export occurring through the Crm1-dependent pathway, we performed export reactions in the presence of the Crm1-specific inhibitor LMB (Fig. 7C). We found that the stimulation of nuclear protein export by NXT1 was completely blocked by 0.5 μM LMB, indicating that NXT1 does, in fact, function in the same export pathway as Crm1.

DISCUSSION

In this study, we used multiple functional assays to characterize NXT1, a protein that we identified based on its sequence relatedness to NTF2. The similarities of NXT1 and NTF2 include their amino acid identity (26% within a species), low molecular sizes (NTF2, 127 amino acids; NXT1, 140 amino acids), acidic isoelectric points (NTF2, 5.1; NXT1, 5.0), steady-state nuclear localization (45), interaction with the NPC (6, 31, 36), and direct binding to Ran (31, 34). However, NXT1 and NTF2 also have distinct properties that provide insights into their respective functions. NTF2 binds to Ran-GDP and mediates its import into the nucleus (38, 43, 45), thereby functioning as a nuclear import factor. In contrast, NXT1 binds to Ran-GTP. The precise function of this interaction is unknown, but it clearly suggests a role in nuclear export. Indeed, using a permeabilized cell assay (16), we have shown here that NXT1 stimulates nuclear export of PKI. The logical interpretation of this result is that NXT1 functions on an export pathway for which Crm1 is the major receptor; this view is corroborated by our finding that the Crm1-specific inhibitor LMB (51) blocks the stimulatory effect of NXT1 in the PKI export assay.

Based on a recent report from Katahira et al. NXT1 also appears to function on at least one pathway of RNA export in the cell (21). After the completion of this study, NXT1 (termed

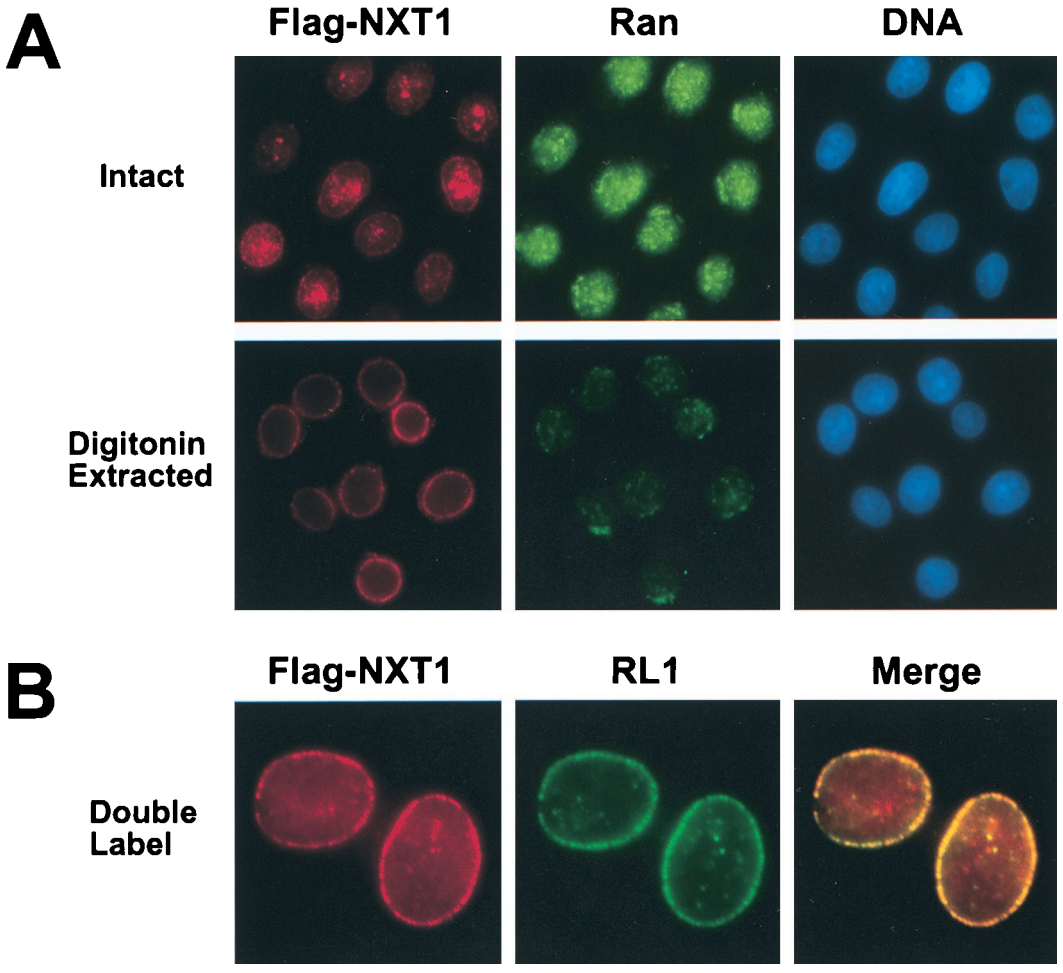

FIG. 5. Subcellular distribution of NXT1. (A) Indirect immunofluorescence microscopy of HeLa cells stably transfected with pFlag-NXT1, showing that NXT1 is a nuclear protein. NXT1 also localizes to the nuclear envelope. After digitonin extraction, NXT1 is released from the nucleoplasm but remains associated with the nuclear envelope (A, lower row). Nuclear Ran is released from the nucleus under these conditions. (B) NXT1 colocalizes with the NPC. Flag-NXT1 cells were labeled with α-Flag antibodies (red) and NPC-specific antibodies (green). The merged image reveals the coincident localization of NXT1 and NPCs (yellow).

p15) was detected as a polypeptide that coimmunoprecipitates with TAP, a protein that stimulates mRNA export when expressed ectopically in the *Xenopus* oocyte (12, 21). Additional evidence that NXT1 and TAP interact was obtained by showing that the proteins can bind to each other when coexpressed in bacteria (21). Mammalian TAP is the probable orthologue of Mex67p, a protein required for mRNA export in yeast (42). Interestingly, while NXT1 is not represented in the yeast genome, another low-molecular-weight protein named Mtr2p for its mRNA trafficking function is present (19). Mtr2p and

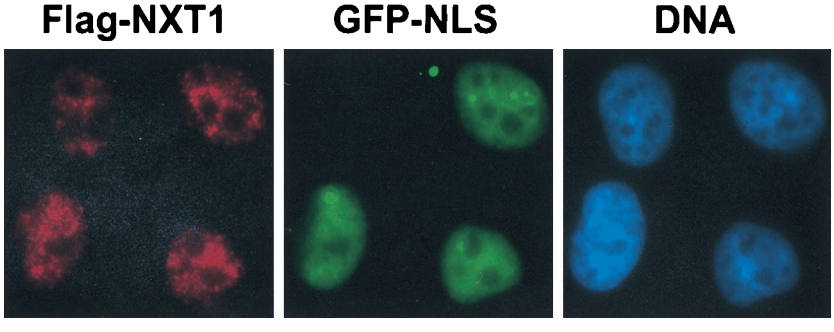

FIG. 6. NXT1 shuttles between the nucleus and cytoplasm in living cells. The Flag-NXT1 cell line was fused with a cell line stably expressing constitutively nuclear GFP-NLS. Heterokaryons were analyzed by immunofluorescence to localize Flag-NXT1 (red) and GFP-NLS (green). In the heterokaryon shown, Flag-NXT1 protein was exported from a single donor nucleus and imported into three acceptor nuclei containing GFP-NLS. NXT1 shuttling was observed in multiple heterokaryons from three separate experiments.

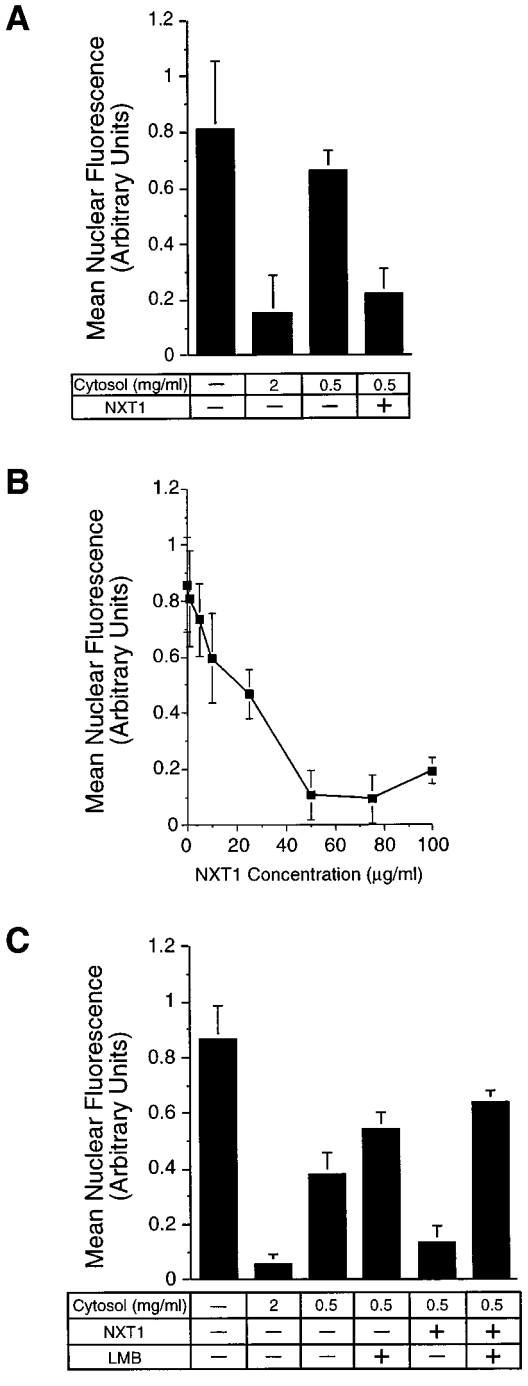

FIG. 7. NXT1 is a nuclear export factor. (A) NXT1 stimulates NES-dependent nuclear export in digitonin-permeabilized cells. Export reactions were performed with HeLa cell cytosol in the absence and presence of recombinant NXT1 (100 μg/ml). The postexport nuclear fluorescence was measured in ~50 randomly selected nuclei by digital fluorescence microscopy and plotted as the mean nuclear fluorescence (±SD) (16). We also found NXT1 could stimulate PKI export in the absence of cytosol (data not shown). (B) Dose-response of NXT1-stimulated nuclear protein export. Export reactions were performed at a subsaturating concentration of cytosol and increasing concentrations of NXT1. We note that NTF2 assayed under similar reaction conditions also stimulates nuclear export, though to a lesser extent (data not shown). The nuclear export stimulation by NTF2 is probably linked to its ability to increase the nuclear concentration of Ran in permeabilized cell nuclei. (C) LMB blocks NXT1-stimulated nuclear protein export. The stimulation of nuclear protein export by NXT1 is blocked in the presence of LMB, a specific inhibitor of Crm1 (51).

Mex67p physically interact, and a double deletion (*mtr2*$^-$ *mex67*$^-$) mutant is inviable (41). These observations suggest that NXT1 and TAP could be the functional counterparts of Mtr2p and Mex67p, respectively. Some evidence supporting this hypothesis was obtained; coexpression of NXT1 and TAP partially rescued the growth defect of the *mtr2*$^-$ *mex67*$^-$ double mutant (21). The extent to which NXT1 and TAP rescued the mRNA export defect was not reported. It is noteworthy that the affinity of TAP for the constitutive transport element of simple retroviruses is nearly 1,000-fold higher than its affinity for dihydrofolate reductase mRNA (3). Thus, since TAP is a sequence-specific mRNA-binding protein (3, 12, 20), higher eukaryotes are predicted to contain additional factors for efficient recognition and transport of mRNA (15). In this regard, it is tempting to speculate that NXT1 interacts with additional cellular factors that mediate export of mRNA.

Our data differ from those of Katahira et al., however, with regard to Ran binding. In their study, recombinant NXT1 was tested for its ability to bind RanGDP or Ran-GTP in pull-down assays, and no interactions were observed (21). In contrast, we used recombinant proteins and found that NXT1 binding to Ran-GTP is saturable and of relatively high affinity (K_D = 8.5 nM). The source of the discrepancy is not known, but it might be related to the fact that the other study used NXT1 protein expressed with an affinity tag, while our recombinant protein was expressed as an unfused polypeptide. Importantly, the recombinant NXT1 used in our binding experiments also stimulates nuclear protein export in digitonin-permeabilized cells. This confirms that the NXT1 protein used in our studies is biochemically active.

The predicted Ran-binding domain of NXT1 is expected to provide a hydrophobic pocket into which Phe72 of Ran can insert its side chain, but how do the NTF2 and NXT1 proteins distinguish between the two nucleotide-bound forms of Ran? NTF2 binding to Ran-GDP is stabilized by a salt bridge between Glu42 (NTF2) and Arg76 (Ran) (46), residues that are invariant among both proteins in all species. The residue corresponding to Glu42 in NXT1 is Asn48, a residue whose conservation among NXT1 proteins implies it could function in stabilizing Ran-GTP binding. Notably, the side chain of Gln69 in the switch II region of Ran provides chemical complementarity to Asn48 in NXT1, indicating how nucleotide-specific binding might be achieved.

The nuclear protein export function of NXT1 is very likely to be coupled to its interaction with Ran-GTP. NXT1 does not modulate the GTPase cycle of Ran under the conditions examined, suggesting that it functions in a noncatalytic manner. NXT1 could, for example, stabilize Ran-GTP binding to a multisubunit export complex containing Crm1 and NES cargo. By analogy, NXT1 might also be expected to target Ran-GTP to a multisubunit export complex containing TAP and mRNA. As a putative subunit of an RNP complex, NXT1 could link the assembly-disassembly regulatory functions of the Ran GTPase to the RNA recognition and transport functions of TAP. An alternative, though not mutually exclusive, possibility is that NXT1 actively targets export complexes to sites within the NPC. This scenario would require that NXT1, like NTF2, interact with multiple nucleoporins (6, 31, 36). NXT1 shuttling between the nucleus and cytoplasm and its localization to the NPC are consistent with targeting export complexes to distinct nucleoporins. It is also possible that NXT1 targets Ran to sites within the NPC. Regulating the local concentration of Ran-GTP within the NPC could influence the assembly state of export complexes or indirectly influence the interaction of export complexes with nucleoporins. We note that other Ran-GTP-binding proteins (RanBP1 and RanBP2) can regulate

nuclear export at the NPC by promoting disassembly and release of Crm1-containing export complexes in a terminal step of transport (22). If NXT1 does have such a role in terminating export, its mechanism of action is likely to differ from that used by RanBP1 and RanBP2. These proteins terminate export by stimulating RanGAP-catalyzed GTP hydrolysis by Ran (22).

In conclusion, our functional analysis of NXT1 has revealed its direct interaction with Ran-GTP, and an important role in the export pathway mediated by Crm1. Our results together with the observed interaction with TAP (21) indicate that NXT1 operates on both protein and RNA export pathways, where it could link the function of export factors to the Ran GTPase cycle. These findings have revealed a surprising convergence of nuclear export pathways, and the continued analysis of these proteins will undoubtedly provide new insights into the pathways of protein and RNA transport.

ACKNOWLEDGMENTS

We thank D. Allis, D. Burke, D. Castle, I. Macara, and B. Pearson for comments on the manuscript. We thank C. Petersen and A. Smith (Macara lab), M. Santisteban, R. Mahajan, and D. Gorlich for gifts of reagents, and S. Zweifel for helpful discussions.

This research was supported by American Cancer Society grant RPG98-048-01-CSM to B.M.P. and by funds from the Lucille P. Markey Charitable Trust.

REFERENCES

1. **Bischoff, F. R., C. Klebe, J. Kretschmer, A. Wittinghofer, and H. Ponstingl.** 1994. RanGAP1 induces GTPase activity of nuclear Ras-related Ran. Proc. Natl. Acad. Sci. USA **91:**2587–2591.
2. **Bischoff, F. R., H. Krebber, E. Smirnova, W. Dong, and H. Ponstingl.** 1995. Co-activation of RanGTPase and inhibition of GTP dissociation by Ran-GTP binding protein RanBP1. EMBO J. **14:**705–715.
3. **Braun, I. C., E. Rohrbach, C. Schmitt, and E. Izaurralde.** 1999. TAP binds to the constitutive transport element (CTE) through a novel RNA-binding motif that is sufficient to promote CTE-dependent RNA export from the nucleus. EMBO J. **18:**1953–1965.
4. **Bullock, T. L., W. D. Clarkson, H. M. Kent, and M. Stewart.** 1996. 1.6 A structure of nuclear transport factor 2 (NTF2). J. Mol. Biol. **260:**422–431.
5. **Chervitz, S. A., L. Aravind, G. Sherlock, C. A. Ball, E. V. Koonin, S. S. Dwight, M. A. Harris, K. Dolinski, S. Mohr, T. Smith, S. Weng, J. M. Cherry, and D. Botstein.** 1998. Comparison of the complete protein sets of worm and yeast: orthology and divergence. Science **282:**2022–2028.
6. **Clarkson, W. D., A. H. Corbett, B. M. Paschal, H. M. Kent, A. McCoy, L. Gerace, P. Silver, and M. Stewart.** 1997. Nuclear protein import is decreased by engineered mutants of nuclear transport factor 2 (NTF2) that do not bind GDP-Ran. J. Mol. Biol. **272:**716–730.
7. **Corbett, A. H., and P. A. Silver.** 1996. The *NTF2* gene encodes an essential, highly conserved protein that functions in nuclear transport in vivo. J. Biol. Chem. **271:**18477–18484.
8. **Dahlberg, J. E., and E. Lund.** 1998. Functions of the GTPase Ran in RNA export from the nucleus. Curr. Opin. Cell Biol. **10:**400–408.
9. **Fitch, W. M., and E. Margoliash.** 1967. Construction of phylogenetic trees. Science **155:**279–284.
10. **Fornerod, M., M. Ohno, M. Yoshida, and I. W. Mattaj.** 1997. CRM1 is an export receptor for leucine-rich nuclear export signals. Cell **90:**1051–1060.
11. **Gorlich, D.** 1998. Transport into and out of the cell nucleus. EMBO J. **17:**2721–2727.
12. **Gruter, P., C. Tabernero, C. Vonkobbe, C. Schmitt, C. Saavedra, A. Bachi, M. Wilm, B. K. Felber, and E. Izaurralde.** 1998. TAP, the human homolog of Mex67p, mediates CTE-dependent RNA export from the nucleus. Mol. Cell **1:**649–659.
13. **Guan, T., S. Muller, G. Klier, N. Pante, J. M. Blevitt, M. Haner, B. Paschal, U. Aebi, and L. Gerace.** 1995. Structural analysis of the p62 complex, an assembly of O-linked glycoproteins that localizes near the central gated channel of the nuclear pore complex. Mol. Biol. Cell **6:**1591–1603.
14. **Guthrie, C., and G. R. Fink (ed.).** 1991. Methods in enzymology, vol. 194. Guide to yeast genetics and molecular biology. Academic Press, New York, N.Y.
15. **Hammarskjöld, M. L.** 1997. Regulation of retroviral RNA export. Semin. Cell Dev. Biol. **8:**83–90.
16. **Holaska, J. M., and B. M. Paschal.** 1998. A cytosolic activity distinct from Crm1 mediates nuclear export of protein kinase inhibitor in permeabilized cells. Proc. Natl. Acad. Sci. USA **95:**14739–14744.
17. **Hu, T. H., T. L. Guan, and L. Gerace.** 1996. Molecular and functional characterization of the p62 complex, an assembly of nuclear pore complex glycoproteins. J. Cell Biol. **134:**589–601.
18. **Izaurralde, E., and S. Adam.** 1998. Transport of macromolecules between the nucleus and the cytoplasm. RNA **4:**351–364.
19. **Kadowaki, T., M. Hitomi, S. Chen, and A. M. Tartakoff.** 1994. Nuclear mRNA accumulation causes nucleolar fragmentation in yeast *mtr2* mutant. Mol. Biol. Cell **5:**1253–1263.
20. **Kang, Y., and B. R. Cullen.** 1999. The human Tap protein is a nuclear mRNA export factor that contains novel RNA-binding and nucleocytoplasmic transport sequences. Genes Dev. **13:**1126–1139.
21. **Katahira, J., K. Straesser, A. Podtelejnikov, M. Mann, J. U. Jung, and E. Hurt.** 1999. The Mex67p-mediated nuclear mRNA export pathway is conserved from yeast to human. EMBO J. **18:**2593–2609.
22. **Kehlenbach, R. H., A. Dickmanns, A. Kehlenbach, T. Guan, L. Gerace.** 1999. A role for RanBP1 in the release of CRM1 from the nuclear pore complex in a terminal step of nuclear transport. J. Cell Biol. **145:**645–657.
23. **Kuhlmann, J., I. Macara, and A. Wittinghofer.** 1997. Dynamic and equilibrium studies on the interaction of Ran with its effector, RanBP1. Biochemistry **36:**12027–12035.
24. **Kutay, U., F. R. Bischoff, S. Kostka, R. Kraft, and D. Gorlich.** 1997. Export of importin alpha from the nucleus is mediated by a specific nuclear transport factor. Cell **90:**1061–1071.
25. **Mahajan, R., C. Delphin, T. Guan, L. Gerace, and F. Melchior.** 1997. A small ubiquitin-related polypeptide involved in targeting RanGAP1 to nuclear pore complex protein RanBP2. Cell **88:**97–107.
26. **Mattaj, I. W., and L. Englmeier.** 1998. Nucleocytoplasmic transport—the soluble phase. Annu. Rev. Biochem. **67:**265–306.
27. **Melchior, F., and L. Gerace.** 1998. Two-way trafficking with Ran. Trends Cell Biol. **8:**175–179.
28. **Michael, W. M., P. S. Eder, and G. Dreyfuss.** 1997. The K nuclear shuttling domain: a novel signal for nuclear import and nuclear export in the hnRNP K protein. EMBO J. **16:**3587–3598.
29. **Moore, M. S., and G. Blobel.** 1994. Purification of a Ran-interacting protein that is required for protein import into the nucleus. Proc. Natl. Acad. Sci. USA **91:**10212–10216.
30. **Murphy, R., and S. R. Wente.** 1996. An RNA-export mediator with an essential nuclear export signal. Nature **383:**357–360.
31. **Nehrbass, U., and G. Blobel.** 1996. Role of the nuclear transport factor p10 in nuclear import. Science **272:**120–122.
32. **Oki, M., and T. Nishimoto.** 1998. A protein required for nuclear-protein import, Mog1p, directly interacts with GTP-Gsp1p, the *Saccharomyces cerevisiae* Ran homologue. Proc. Natl. Acad. Sci. USA **95:**15388–15393.
33. **Parker, F., F. Maurier, I. Delumeau, M. Duchesne, D. Faucher, L. Debussche, A. Dugue, F. Schweighoffer, and B. Tocque.** 1996. A Ras-GTPase-activating protein SH3-domain-binding protein. Mol. Cell. Biol. **16:**2561–2569.
34. **Paschal, B. M., C. Delphin, and L. Gerace.** 1996. Nucleotide-specific interaction of Ran/TC4 with nuclear transport factors NTF2 and p97. Proc. Natl. Acad. Sci. USA **93:**7679–7683.
35. **Paschal, B. M., C. Fritze, T. Guan, and L. Gerace.** 1997. High levels of the GTPase Ran/TC4 relieve the requirement for nuclear protein transport factor 2. J. Biol. Chem. **272:**21534–21539.
36. **Paschal, B. M., and L. Gerace.** 1995. Identification of NTF2, a cytosolic factor for nuclear import that interacts with nuclear pore complex protein p62. J. Cell Biol. **129:**925–937.
37. **Pearson, W. R., T. Wood, Z. Zhang, and W. Miller.** 1997. Comparison of DNA sequences with protein sequences. Genomics **46:**24–36.
38. **Ribbeck, K., G. Lipowsky, H. M. Kent, M. Stewart, and D. Gorlich.** 1998. NTF2 mediates nuclear import of Ran. EMBO J. **17:**6587–6598.
39. **Richards, S. A., K. L. Carey, and I. G. Macara.** 1997. Requirement of guanosine triphosphate-bound Ran for signal-mediated nuclear protein export. Science **276:**1842–1844.
40. **Saitoh, H., R. Pu, M. Cavenagh, and M. Dasso.** 1997. RanBP2 associates with Ubc9p and a modified form of RanGAP1. Proc. Natl. Acad. Sci. USA **92:**3736–3741.
41. **Santos-Rosa, H., H. Moreno, G. Simos, A. Segref, B. Fahrenkrog, N. Pante, and E. Hurt.** 1998. Nuclear mRNA export requires complex formation between Mex67p and Mtr2p at the nuclear pores. Mol. Cell. Biol. **18:**6826–6838.
42. **Segref, A., K. Sharma, V. Doye, A. Hellwig, J. Huber, R. Luhrmann, and E. Hurt.** 1997. Mex67p, a novel factor for nuclear mRNA export, binds to both poly(A)$^+$ RNA and nuclear pores. EMBO J. **16:**3256–3271.
43. **Smith, A., A. Brownawell, and I. G. Macara.** 1998. Nuclear import of Ran is mediated by the transport factor NTF2. Curr. Biol. **8:**1403–1406.
44. **Snow, C. M., A. Senior, and L. Gerace.** 1987. Monoclonal antibodies identify a group of nuclear pore complex glycoproteins. J. Cell Biol. **104:**1143–1156.
45. **Steggerda, S. M., and B. M. Paschal.** Monoclonal antibodies to NTF2 inhibit nuclear protein import by preventing nuclear translocation of the GTPase Ran. Submitted for publication.
46. **Stewart, M., H. M. Kent, and A. J. McCoy.** 1998. Structural basis for molecular recognition between nuclear transport factor 2 (NTF2) and the GDP-bound form of the Ras-family GTPase Ran. J. Mol. Biol. **277:**635–646.
47. **Strambio de Castillia, C., and M. P. Rout.** 1999. TAPping into transport. Nat. Cell Biol. **1:**E31–E33.

48. **Tachibana, T., M. Hieda, T. Sekimoto, and Y. Yoneda.** 1996. Exogenously injected nuclear import factor p10/NTF2 inhibits signal-mediated nuclear import and export of proteins in living cells. FEBS Lett. **397:**177–182.
49. **Vetter, I. R., C. Nowak, T. Nishimoto, J. Kuhlmann, and A. Wittinghofer.** 1999. Structure of a Ran-binding domain complexed with Ran bound to a GTP analogue: implications for nuclear transport. Nature **398:**39–46.
50. **Watkins, J. L., R. Murphy, J. L. T. Emtage, and S. R. Wente.** 1998. The human homologue of *Saccharomyces cerevisiae* Gle1p is required for poly(A)$^+$ RNA export. Proc. Natl. Acad. Sci. USA **95:**6779–6784.
51. **Wolff, B., J. J. Sanglier, and Y. Wang.** 1997. Leptomycin B is an inhibitor of nuclear export: inhibition of nucleo-cytoplasmic translocation of the human immunodeficiency virus type 1 (HIV-1) Rev protein and Rev-dependent mRNA. Chem. Biol. **4:**139–147.
52. **Wong, D. H., A. H. Corbett, H. M. Kent, M. Stewart, and P. A. Silver.** 1997. Interaction between the small GTPase Ran/Gsp1p and Ntf2p is required for nuclear transport. Mol. Cell. Biol. **17:**3755–3767.