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Nucleotide-specific interaction of Ran/TC4 with nuclear transport factors NTF2 and p97

(GTPase/nuclear pore complex/protein import)

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ABSTRACT **The use of permeabilized cell models to study nuclear protein import has led to the identification of cytosolic components of the import machinery, including the NLS receptor, p97, Ran/TC4, and nuclear transport factor 2 (NTF2). These proteins are required to reconstitute docking of transport ligand at the nuclear pore complex and subsequent translocation through the nuclear pore. However, a detailed molecular understanding of how these factors mediate protein import is lacking. Here we describe the results of solution and solid phase binding assays, which demonstrate that the small GTPase Ran/TC4 interacts directly with the cytosolic transport factors p97 and NTF2. By preloading recombinant Ran/TC4 with [γ-^{32}P]GTP or [^{3}H]GDP, we show that the interactions with p97 and NTF2 are specific for the GTP- and GDP-bound forms, respectively. These data together with previous studies lead us to suggest that the interaction of the GTP-bound form of Ran/TC4 with p97 is linked to an early step in the nuclear protein import pathway and that the association of the GDP-bound form of Ran/TC4 with NTF2 helps define vectorial transport.**

The integration of nuclear functions with biosynthetic and signaling events in the cytoplasm requires rapid and selective nucleocytoplasmic transport. Molecular trafficking between the nuclear and cytoplasmic compartments is mediated by large supramolecular structures that span the nuclear envelope known as nuclear pore complexes (NPCs; reviewed in refs. 1–3). Ions and small molecules cross the NPC by passive diffusion, while most proteins and ribonucleoprotein complexes are transported by signal- and ATP-dependent mechanisms. There are multiple steps in the nuclear protein import pathway, including the docking of a nuclear localization sequence (NLS)-containing protein at the cytoplasmic periphery of the NPC, accumulation near the central gated channel, and translocation into the nucleus (reviewed in refs. 4 and 5).

The development of simple, quantitative assays for measuring nuclear protein import *in vitro* (6, 7) has led to the identification of several cytosolic factors that play direct roles in this process. These factors† are the NLS receptor (8–11), p97 (12–15), Ran/TC4 (17, 18), and nuclear transport factor 2 (NTF2; refs. 7 and 16). One of the earliest events in the protein import pathway occurs when the NLS receptor binds to the NLS of a protein ligand destined for import and mediates ligand docking at the cytoplasmic periphery of the NPC in a complex with p97 (reviewed in ref. 5). The function of p97 in this step of transport seems to be that of an adaptor protein, which binds directly to both the NLS receptor and to the NPC (12–15, 19). While addition of the NLS receptor and p97 is sufficient to reconstitute docking of NLS ligand in permeabilized cells, Ran/TC4 and NTF2 must also be present to reconstitute delivery of the NLS ligand into the nucleoplasm (7, 16). How Ran/TC4 and NTF2 facilitate transport steps downstream of docking is unknown, though the fractionation behavior of the *Xenopus* homologues of these proteins leads to the suggestion they may interact with each other (16). It is clear that obtaining a detailed understanding of nuclear protein import requires defining how the cytosolic transport factors interact with each other and with proteins of the NPC to mediate discrete transport steps.

A number of NPC proteins have been characterized in vertebrates, among them a group of O-linked glycoproteins whose primary structures contain degenerate repeats involving the dipeptide sequence FG (phenylalanine-glycine; reviewed in ref. 3). The O-linked glycoproteins appear to have a direct role in nuclear import, since the binding of wheat germ agglutinin and monoclonal antibodies to them inhibits nuclear protein import, and NPCs assembled in their absence are transport-deficient (reviewed in ref. 1). Furthermore, our laboratory found that this group of polypeptides can specifically deplete cytosol of an *N*-ethylmaleimide-insensitive activity required for protein import (20). We further found that this depletion could be partly ascribed to one of these O-linked glycoproteins, p62 (7). The activity that restores transport to cytosol pretreated with immobilized p62 was purified. This activity is NTF2, a low molecular weight homodimeric protein (subunit mass = 14,478 Da) that seems to be conserved in phylogenetically diverse species (7).

Here we describe the results of a biochemical screen for cytosolic factors that interact with NTF2. We found that the nuclear transport factors Ran/TC4 and p97 were specifically depleted from HeLa cell cytosol by treatment with NTF2-Sepharose. Using purified recombinant proteins, we show that NTF2 interacts directly with the GDP-bound form of Ran/TC4, while p97 interacts directly with the GTP-bound form of Ran/TC4. Together with previous studies, our data suggests that Ran/TC4 is required for at least two distinct steps in the pathway of nuclear protein import which are defined by its nucleotide state.‡

MATERIALS AND METHODS

Preparation of HeLa Cell Cytosol and Affinity Depletion of Transport Factors. A high-speed cytosolic extract (150,000 × *g* supernatant fraction) was prepared from HeLa cells grown in suspension culture as described (7). The concentration of

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Abbreviations: NLS, nuclear localization sequence; NPC, nuclear pore complex; NTF2, nuclear transport factor 2; GST, glutathione *S*-transferase; RanBP2, Ran/TC4 binding protein 2.

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†The NLS receptor has also been referred to as importin α, karyopherin α, and SRP1 (8–11). The protein p97 has also been referred to as importin β and karyopherin β (12–15). NTF2 has also been referred to as p10 (7, 16).

‡The results of this study were presented at the 35th Annual Meeting of the American Society for Cell Biology and published in abstract form (21).

protein in the cytosolic extract was ≈10 mg/ml, in a buffer containing 20 mM Hepes (pH 7.4), 110 mM potassium phosphate, 2 mM magnesium acetate, 0.5 mM EGTA, and 2 mM DTT (transport buffer). The matrix for affinity depletion was prepared by coupling recombinant human NTF2 to CNBr-activated Sepharose (Pharmacia LKB) at a concentration of ≈2 mg of protein per ml of beads. After coupling the beads were quenched with 1 M ethanolamine, washed extensively with transport buffer, and transferred to a 10-ml disposable column. The column was treated with several volumes of 100 mg of BSA per ml to block nonspecific binding and washed again with transport buffer. Cytosol was passed over the column (volume ratio 1:1) three times, the unbound fraction was concentrated by vacuum dialysis, frozen in liquid N_2, and stored at −80°C. The depletion of known transport factors was examined with antibodies against p97 (13), SRP1 (11), and Ran/TC4 (22). A rabbit antibody to human NTF2 was prepared by using the recombinant protein both as the antigen and as the substrate for affinity purification.

Solution Binding of NTF2 and Ran/TC4. Human NTF2 and Ran/TC4 were expressed in bacteria and purified as described (7, 17). Based on the basal GTP hydrolysis rate, the recombinant Ran/TC4 should be a mixture of GTP- and GDP-bound forms, the latter constituting ≈80% of the total protein. Recombinant proteins (stored at −80°C) were thawed and clarified for 5 min in an air-driven ultracentrifuge (28 psi). Samples containing Ran/TC4 alone, NTF2 alone, or a mixture of the two proteins (25 μg each) were incubated on ice for 60 min in transport buffer containing 5 mM DTT and 100 μg of BSA in a total volume of 200 μl. Chromatography was performed in transport buffer on a Superdex 75 FPLC column at a flow rate of 0.5 ml/min. The elution profiles of the proteins were examined by immunoblotting with an anti-peptide antibody specific for Ran/TC4 (22) and with a monoclonal antibody (Novagen) reacting with the N-terminal T7 epitope tag on recombinant NTF2.

Chemical Crosslinking of NTF2 and Ran/TC4. The crosslinking reactions involved NTF2 and Ran/TC4, each at a final concentration of 100 μg/ml in transport buffer. The proteins were incubated on ice for 60 min, and the crosslinker disuccinyl suberate (Pierce) was added from a 20 mM stock (in DMSO) to a final concentration of 0.2 mM. The reactions were quenched by the addition of excess Tris·Cl, and the products were analyzed by immunoblotting. Note the blot containing the crosslinked products of Ran/TC4 and NTF2 was probed, stripped, and reprobed with the relevant antibodies.

Determination of the Nucleotide State of Ran/TC4 That Interacts with NTF2. Recombinant Ran/TC4 (25 μg) was loaded with 20 μM guanine nucleotide ([^{3}H]GDP or [γ-^{32}P]GTP) in the presence of 10 mM EDTA, 2.5 mM DTT, 2 mM ATP, and 15 mM Hepes (pH 7.4) in a total volume of 50 μl for 20 min at 30°C. The reaction was diluted with excess magnesium ions and chromatographed on a PD-10 gel filtration column (Pharmacia LKB) to remove unbound nucleotide. The nucleotide-loaded Ran/TC4 (10 μg) was incubated in the absence or presence of NTF2 (50 μg) along with BSA (2 mg) for 60 min on ice. The samples were chromatographed on a Sephacryl S100 HR column (40 ml) at a flow rate of 20 ml/hr in transport buffer. The elution position of the radiolabeled GDP- or GTP-Ran/TC4 was then determined by scintillation counting 50 μl of each 1-ml fraction. The components of the nucleotide exchange reactions were Ran/TC4 (1.2 μg) preloaded with [^{3}H]GDP, RCC1 (2.8 μg), NTF2 (16 μg), and 2 mM GTP in a total volume of 100 μl. The reactions (duplicate points) were performed in transport buffer at 30°C for 10 min. [^{32}P]GTP, which remained bound to Ran/TC4, was quantitated by a nitrocellulose binding assay.

Binding Assays Featuring Glutathione *S*-Transferase (GST)-Fusions of Ran/TC4 and p97. Human Ran/TC4 and rat p97 were expressed as N-terminal GST fusions in bacteria and immobilized on glutathione beads at a protein concentration of 1–5 mg/ml. Soluble recombinant Ran/TC4 (5 μg), NTF2 (5 μg), or both were mixed with 20 μl of packed beads in a total volume of 200 μl for 60 min at 4°C. The assays were performed in transport buffer containing 2.5 mM DTT and included 100 μg of BSA as a blocking agent. The beads were recovered by centrifugation and washed four times in transport buffer, and the bound proteins were eluted and detected by immunoblotting. To detemine the nucleotide state of Ran/TC4 that interacts with p97, we preloaded Ran/TC4 with radiolabled GDP or GTP as described above and carried out binding reactions (triplicate points) with immobilized GST or GST-p97. The bound fractions were eluted with 5% SDS/20 mM EDTA and assayed by scintillation counting.

RESULTS

We showed previously that HeLa cell cytosol pretreated with Sepharose-immobilized NTF2 was strongly deficient (≈30% of control) in its ability to support protein import in the permeabilized cell assay, indicating that NTF2 interacts with additional soluble factors (7). The addition of individual cytosolic fractions from sucrose density gradients failed to restore transport to cytosol pretreated with NTF2-Sepharose, suggesting this procedure may have removed more than one rate-limiting factor (B.M.P., unpublished observations). To determine whether NTF2-Sepharose removed any of the previously characterized transport factors, the unbound fractions of cytosol treated in this manner were examined by immunoblotting (Fig. 1). We found that the levels of p97 and Ran/TC4 in cytosol were drastically reduced under these conditions, while the levels of the NLS receptor and NTF2 were not detectably changed (Fig. 1).

To determine whether the depletion of cytosolic Ran/TC4 was due to a direct interaction with NTF2, binding experiments using recombinant forms of both proteins were performed (Fig. 2). Purified preparations of recombinant Ran/TC4 and NTF2 eluted from a Superdex 75 FPLC column with apparent molecular weights of ≈25 and ≈30 kDa, respectively (Fig. 2*A*, *Top* and *Middle*). This agrees with the apparent native sizes of

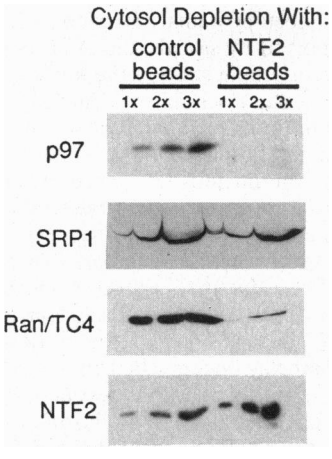

FIG. 1. Affinity depletion of transport factors from HeLa cell cytosol using Sepharose-immobilized NTF2. Cytosol was passed three times over Sepharose beads coupled with either 2 mg of BSA per ml or 2 mg of recombinant NTF2 per ml (5). The relative levels of the nuclear protein import factors [p97, SRP1 (NLS receptor), Ran/TC4, and NTF2] in the unbound fraction were then examined by immunoblotting. For comparative purposes, 10, 20, and 30 μl (denoted 1x, 2x, and 3x) of the unbound fraction from each column was analyzed. The transport factors p97 and Ran/TC4 were effectively removed from cytosol by this treatment, while the levels of the NLS receptor and NTF2 were relatively unchanged. Note that cytosol treated in this manner supports transport at ≈30% of control cytosol (7).

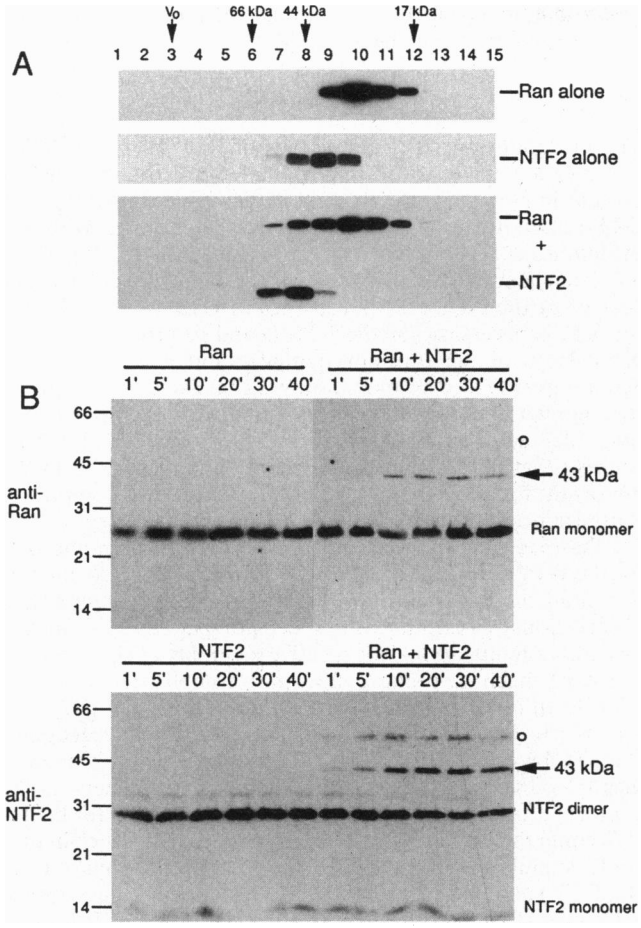

FIG. 2. Direct interaction of NTF2 with the GTPase Ran/TC4. (*A*) Purified recombinant Ran/TC4 and NTF2 alone and in combination were chromatographed on a Superdex 75 FPLC column and detected by immunoblotting. The two proteins form a complex that elutes with an apparent molecular mass of 44 kDa. (*B*) Purified recombinant NTF2 and Ran/TC4 were crosslinked using disuccinimidyl suberate for the indicated times, and the products were analyzed by immunoblotting. Crosslinking a mixture of NTF2 and Ran/TC4 resulted in the formation of a 43-kDa species with a predicted stoichiometry of 1:1 (NTF2:Ran/TC4). The ≈55-kDa product generated by crosslinking (open circle) was recognized by the NTF2 antibody (*Lower*) but not by the Ran/TC4 antibody (*Upper*).

Ran/TC4 and the NTF2 homodimer. Gel filtration analysis of a mixture of the two proteins resulted in the appearance of higher molecular weight species of both NTF2 and Ran/TC4, suggesting the formation of a complex *in vitro* (Fig. 2*A*, *Bottom*). While Ran/TC4 eluted in fractions 9–12 when chromatographed alone, it appeared also in fractions 7 and 8 when combined with NTF2. The elution position of NTF2 was altered as well, as the actual peak shifted from fraction 9 to fraction 8. Interestingly, the peak fraction of the complex (fraction 8) elutes with an apparent molecular mass of ≈40 kDa, which could reflect the binding of Ran/TC4 to an NTF2 monomer.

Further evidence for the formation of a Ran/TC4-NTF2 complex was obtained in chemical crosslinking experiments. Recombinant proteins alone and in combination were treated with the crosslinker disuccinyl suberate for up to 40 min, and the products were examined by immunoblotting with monospecific antibodies. Ran/TC4 alone remained a monomeric species during the entire time course (Fig. 2*B*, *Upper*), whereas NTF2 alone was nearly quantitatively crosslinked into a dimer during the first minute of incubation (*Lower*). Crosslinking of a mixture of the proteins generated an ≈43-kDa species, which was recognized by antibodies to both Ran/TC4 and epitope-tagged NTF2. These conditions also produced an ≈60-kDa species (indicated by the open circle), which was recognized by the anti-NTF2 antibody but not by the anti-Ran/TC4 antibody. The 60-kDa product may be a tetramer of NTF2, or it could represent a product composed of one copy of Ran/TC4 and a dimer of NTF2 that is not recognized by our anti-Ran/TC4 antibodies (22).

We then examined whether the binding of NTF2 to Ran/TC4 is nucleotide-specific. In this experiment, Ran/TC4 was preloaded with either [^{3}H]GDP or [^{32}P]GTP and chromatographed on a S100 gel filtration column in the absence and presence of NTF2 (Fig. 3). A size shift of the [^{3}H]GDP-bound form of Ran/TC4 from ≈25 kDa to ≈40 kDa was observed when chromatography was carried out in the presence of NTF2 (Fig. 3*A*). In contrast, the [^{32}P]GTP-bound form of Ran/TC4 eluted at the same position whether chromatographed in the absence or presence of NTF2 (Fig. 3*B*). These data clearly demonstrate that NTF2 binds stably to the GDP-bound form of Ran/TC4 under conditions where no interaction with the GTP-bound form of Ran/TC4 is measured.

The interaction of NTF2 with the GDP-bound form of Ran/TC4 could serve to stabilize this state and consequently

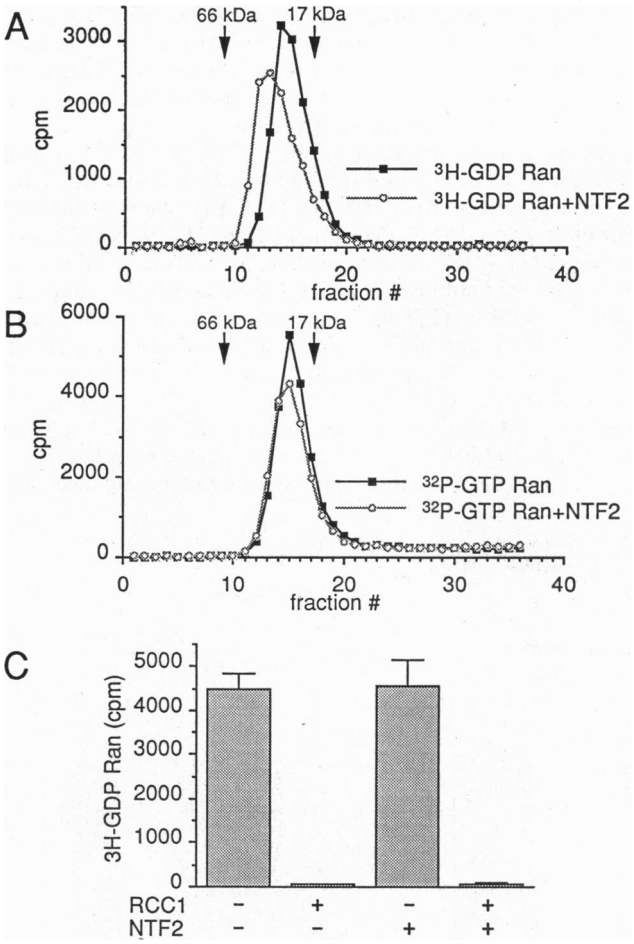

FIG. 3. Interaction of NTF2 with the GDP-bound form of Ran/TC4. (*A*) Recombinant Ran/TC4 was preloaded with [^{3}H]GDP and chromatographed in the absence or presence of recombinant NTF2. Under these conditions, GDP-Ran/TC4 displays a size shift from ≈25 to ≈40 kDa when chromatographed in the presence of NTF2. (*B*) Recombinant Ran/TC4 was preloaded with [^{32}P]GTP and chromatographed in the absence or presence of recombinant NTF2. The elution position of GTP-Ran/TC4 is unaffected by the presence of NTF2. (*C*) The nucleotide exchange activity of recombinant RCC1 was measured (23) in the absence and presence of NTF2. The exchange activity was not detectably affected by NTF2.

act as a guanine nucleotide dissociation inhibitor. Catalyzed nucleotide exchange on the GTPase rho can be inhibited by its guanine nucleotide dissociation inhibitor (23). We therefore tested whether NTF2 could inhibit the nucleotide exchange on Ran/TC4 catalyzed by its exchange factor RCC1 (24), using Ran/TC4 preloaded with [^{3}H]GDP and a filter binding assay. This showed that the RCC1-mediated exchange of GTP for bound GDP was not detectably inhibited, even when carried out in the presence of a 10-fold molar excess of NTF2 (Fig. 3*C*). We also found that the dissociation of bound nucleotide with 10 mM EDTA was affected only slightly by including NTF2 in the reaction (B.M.P., unpublished observations). Thus, it seems unlikely that the function of NTF2 is to protect Ran/TC4 from nucleotide exchange.

The depletion of p97 from HeLa cell cytosol with NTF2-Sepharose (Fig. 1) suggested that NTF2, or perhaps a complex of NTF2 and Ran/TC4, might interact with p97. To probe for interactions between these proteins, we immobilized GST fusions of p97 and Ran/TC4 on Sepharose and tested whether soluble NTF2, Ran/TC4, or a combination of these proteins could bind to these matrices. As expected, NTF2 bound to Ran/TC4, but it did not bind to p97 either in the absence or presence of Ran/TC4. This suggests that the cytosolic depletion of p97 obtained using NTF2-Sepharose (Fig. 1) is mediated by an additional soluble factor. However, it remains possible that the use of recombinant proteins in the binding experiment does not reconstitute an interaction that is observed in cytosol with native proteins.

An interesting and unexpected finding in this experiment was that Ran/TC4 bound directly to p97 and that this interaction was independent of NTF2 (Fig. 4*A*). We reasoned that this interaction, like the binding of Ran/TC4 to NTF2, might be regulated by the nucleotide state. To address this issue, we measured the binding of Ran/TC4 preloaded with radioactive GDP or GTP to GST-p97 immobilized on Sepharose beads (Fig. 4). Only nonspecific binding of [^{3}H]GDP-Ran/TC4 to GST-p97 (23.8 ± 6.2 ng) was obtained, as this was comparable to the binding to GST alone (17.2 ± 11.6 ng). In contrast, the level of [^{32}P]GTP-Ran/TC4 binding to GST-p97 was substantially higher (1204 ± 16.08 ng) as compared with binding to GST alone (7.0 ± 3.1 ng). These data demonstrate a specific, direct interaction of the GTP-bound form of Ran/TC4 with the cytosolic transport factor p97.

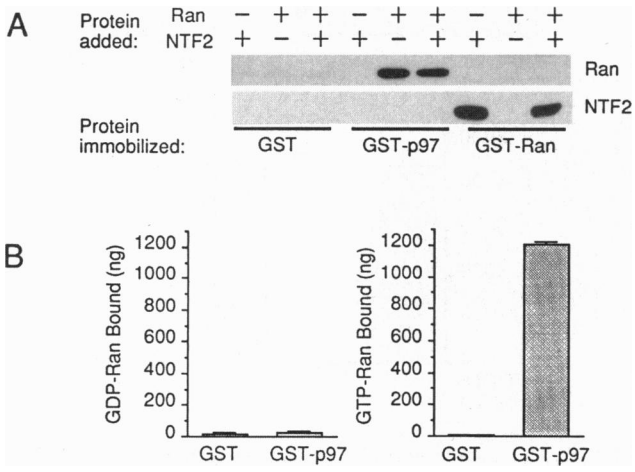

FIG. 4. Direct interaction of cytosolic transport factors NTF2 and p97 with Ran/TC4. (*A*) GST fusions of p97 and Ran/TC4 were expressed in *E. coli*, recovered on glutathione beads, and used for binding assays (25). Ran/TC4 bound specifically to immobilized p97, and NTF2 bound specifically to immobilized Ran/TC4. (*B*) GST and GST-p97 were incubated with recombinant Ran/TC4 preloaded with either [^{3}H]GDP (specific activity, 5895 cpm/μg) or [γ-^{32}P]GTP (specific activity, 10^6 cpm/μg). The interaction of p97 with Ran/TC4 is specific for the GTP-bound form of the latter protein.

DISCUSSION

The nucleotide-specific interactions of Ran/TC4 with NTF2 and p97 are likely to be important features of the nuclear protein import pathway. Recent studies have shown that the GTP-bound form of Ran/TC4 directly interacts with the protein Ran/TC4 binding protein 2 (RanBP2) (25, 26), which is located near the initial ligand docking site on the cytoplasmic surface of the NPC (22). Hydrolysis of GTP by Ran/TC4 at this site is necessary for the NLS ligand to proceed to more distal steps of the transport pathway and may represent a "commitment" step in the pathway (22). These observations, together with our new data showing mutually exclusive nucleotide dependent binding of Ran/TC4 to p97 and NTF2, suggest that these nucleotide-specific interactions may be important for the assembly and dissociation of a complex of these transport factors at RanBP2 (Fig. 5).

It has been shown that RanBP2 contains four binding sites for the GTP-form of Ran/TC4 (25, 26). RanBP2 also appears to contain binding sites for p97 and NTF2 (ref. 27; unpublished observations), prompting us to speculate that this NPC protein provides a template for cooperative assembly of the cytosolic transport machinery into a transport complex (Fig. 5). The GTP form of Ran/TC4 could facilitate binding of p97/NLS receptor to RanBP2 (step 1). Furthermore, the interaction of Ran/TC4 with p97 could allow GTP hydrolysis to be communicated to the p97/NLS receptor/ligand complex (step 2). The conformational change in Ran/TC4 induced by GTP hydrolysis could then abrogate its interaction with p97, which in turn could stimulate dissociation of the transport complex from RanBP2 (step 3). NTF2 could play several roles in these events. NTF2 could augment release of the GDP form of Ran/TC4 from p97, or promote the dissociation of a multisubunit transport complex from RanBP2 (step 3; see legend of Fig. 5). In addition, assembly of the heterodimer containing NTF2 and GDP-Ran/TC4 into the transport complex could provide a molecular marker that specifies vectorial transport, perhaps for targeting the transport complex to subsequent steps in the pathway (ref. 4 and below).

We have shown previously that NTF2 binds directly to NPC protein p62, an interaction which requires the amino terminal half of p62 (7). This region of p62 is notable because it contains multiple FXFG repeats, a feature shared by other O-linked NPC glycoproteins (reviewed in refs. 3 and 4). Indeed, at least nine different O-linked NPC glycoproteins released from nuclear envelopes by detergent and high salt are bound by Sepharose-immobilized NTF2 (unpublished observations). While it remains to be established whether these all represent direct interactions, our laboratory has determined that NTF2 binds directly to recombinant forms of NPC proteins p62 (7) as well as p58 and p54 (T. Hu and L.G, unpublished data). Blot overlay (27) and solution binding (T. Hu and L.G., unpublished data) experiments have shown that p97 also interacts directly with multiple NPC O-linked glycoproteins. The interaction of NTF2 and p97 with the same subset of O-linked glycoproteins, all of which contain FXFG repeats, provides a clue about the mechanism of protein import. Since the O-linked glycoproteins are localized at various cytoplasmic and nucleoplasmic regions of the NPC, p97 and NTF2 may function at multiple steps in the import pathway. Forward progress of the transport complex (after dissociation from RanBP2; Fig. 5) could rely on cooperative and/or sequential interactions of p97 and NTF2 with O-linked glycoproteins in the NPC.

Whether Ran/TC4 serves to stimulate assembly and/or disassembly of transport complexes at steps downstream of RanBP2 is presently unknown. The GTP-form of Ran/TC4 was recently shown to stimulate the release of NLS ligand from

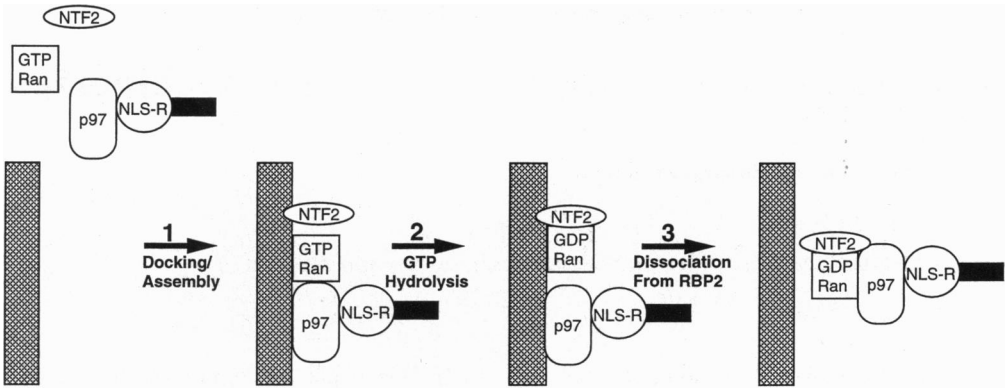

FIG. 5. Hypothetical model depicting interactions of the cytosolic transport factors Ran/TC4, NTF2, NLS receptor (NLS-R), p97, NLS-containing transport ligand (black), and RanBP2 (stippled) during early transport steps at the NPC. The interaction of p97 with NTF2 and the GDP form of Ran/TC4 (step 3) is speculative and has not been reconstituted with recombinant proteins. However, the fact that Ran/TC4 and p97 are depleted from cytosol using NTF2-Sepharose suggests that these proteins interact, either directly or indirectly.

an NLS receptor/p97 heterodimer (28). However, since the addition of an FXFG-containing protein (Nup1) was also reported to release NLS ligand from this complex (28), the implications of these findings remain to be determined. Defining the polypeptide composition of the transport complex, which ultimately translocates through the NPC, remains one of the key questions related to understanding nuclear protein import.

In summary, we have demonstrated that the GTP- and GDP-bound forms of Ran/TC4 interact directly with the cytosolic transport factors p97 and NTF2, respectively. These nucleotide-specific interactions could promote the assembly of a multisubunit transport complex and its dissociation from RanBP2. We envision that the transport factors p97 and NTF2 play dual roles in the import pathway, those of modulating the assembly of the complex and its subsequent targeting to distinct sites in the NPC.

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