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RanGTP Targets p97 to RanBP2, a Filamentous Protein Localized at the Cytoplasmic Periphery of the Nuclear Pore Complex

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RanBP2, a protein containing FG repeat motifs and four binding sites for the guanosine triphosphatase Ran, is localized at the cytoplasmic periphery of the nuclear pore complex (NPC) and is believed to play a critical role in nuclear protein import. We purified RanBP2 from rat liver nuclear envelopes and examined its structural and biochemical properties. Electron microscopy showed that RanBP2 forms a flexible filamentous molecule with a length of ~36 nm, suggesting that it comprises a major portion of the cytoplasmic fibrils implicated in initial binding of import substrates to the NPC. Using in vitro assays, we characterized the ability of RanBP2 to bind p97, a cytosolic factor implicated in the association of the nuclear localization signal receptor with the NPC. We found that RanGTP promotes the binding of p97 to RanBP2, whereas it inhibits the binding of p97 to other FG repeat nucleoporins. These data suggest that RanGTP acts to specifically target p97 to RanBP2, where p97 may support the binding of an nuclear localization signal receptor/substrate complex to RanBP2 in an early step of nuclear import.

INTRODUCTION

In eukaryotic cells, molecular transport between the cytoplasm and the nucleus is mediated by the nuclear pore complex (NPC), a supramolecular structure of ~125 MDa that spans the nuclear envelope. The framework of the NPC consists of nucleoplasmic and cytoplasmic rings flanking eight central spokes, which surround a gated channel involved in the signal-mediated transport of macromolecules (Hinshaw *et al.*, 1992). In addition, eight fibrils, 30–50 nm in length, emanate from the cytoplasmic ring (Ris, 1991; Jarnik and Aebi, 1991; Goldberg and Allen, 1993), and eight fibrils, 50–100 nm in length, extend into the nucleus from the nucleoplasmic ring (for review see Panté and Aebi, 1996a). Metabolites and small molecules cross the NPC by passive diffusion, whereas most macromolecules appear to be transported by signal-dependent, active processes. The signals that specify nuclear protein import (nuclear localization sequences or NLS) are most commonly short stretches of basic amino acid sequences (for review see Dingwall and Laskey, 1991), although at least one additional class of protein NLS is known to exist (Michael *et al.*, 1995; Pollard *et al.*, 1996).

The nuclear import of proteins containing basic type NLS has been characterized in considerable detail (for reviews see Melchior and Gerace, 1995; Görlich and Mattaj, 1996; Panté and Aebi, 1996a; Nigg, 1997). It is a multistep process that involves the binding or "docking" of transport substrates at the cytoplasmic fibrils of the NPC, followed by their movement to the central gated channel and translocation into the nuclear interior. These events are orchestrated by interactions between soluble import factors and components of the NPC. Nuclear protein import in digitonin-permeabilized mammalian cells can be reconstituted with four cytosolic factors: the NLS receptor, p97, Ran, and NTF2. The NLS receptor (also called importin α and karyopherin α) recognizes NLS-containing import substrates in the cytosol and carries them into the nucleus; after dissociation of the substrate in the nucleus, the NLS receptor is subsequently recycled back to the cytoplasm (reviewed in Sweet and Gerace, 1995;

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Görlich and Mattaj, 1996; Nigg, 1997). The association of the cytosolic NLS receptor/substrate complex with the NPC during the import process appears to involve p97 (also known as importin β and karyopherin β), which directly interacts with the NLS receptor (Adam and Adam, 1994; Gorlich *et al.*, 1994; Chi *et al.*, 1995; Görlich *et al.*, 1995a, 1995b) as well as with a number of NPC proteins containing FG (phenylalanine, glycine) repeats (Iovine *et al.*, 1995; Kraemer *et al.*, 1995; Moroianu *et al.*, 1995b; Radu *et al.*, 1995b; Hu *et al.*, 1996). FG repeat nucleoporins are localized in many regions of the NPC and are thought to represent sites for the binding of the substrate/NLS receptor complex during its progressive movement from the cytoplasmic to the nuclear side of the NPC.

The small guanosine triphosphatase Ran/TC4 (Melchior *et al.*, 1993; Moore and Blobel, 1993) and NTF2/p10 (Moore and Blobel, 1994; Paschal and Gerace, 1995) are suggested to promote the vectorial movement of the transport substrate/NLS receptor complex through the NPC, but the functions of these proteins are not well understood. Ran acts as a molecular switch that cycles between guanosine triphosphate (GTP)- and guanosine diphosphate (GDP)-bound forms. The nucleotide state of Ran is regulated by its guanine nucleotide exchange factor RCC1 (Ohtsubo *et al.*, 1987; Bischoff and Ponstingl, 1991a, 1991b), and its guanosine triphosphatase-activating protein RanGAP1 (Bischoff *et al.*, 1994, 1995a). RCC1 is localized in the nucleoplasm (Ohtsubo *et al.*, 1989), whereas RanGAP1 is localized in the cytosol and on the cytoplasmic surface of the NPC (Matunis *et al.*, 1996; Mahajan *et al.*, 1997). The differential nucleocytoplasmic localizations of these two regulators is likely to be of key importance for the vectoriality of the nuclear import pathway.

Ran binds to three additional cytosolic proteins that may contribute to its transport functions: RanBP1, NTF2, and p97. The GTP-bound form of Ran binds to p97 (Rexach and Blobel, 1995; Floer and Blobel, 1996) as well as to RanBP1 (Bischoff *et al.*, 1995b), a 28-kDa protein that is suggested to have a direct role in nucleocytoplasmic transport (Schlenstedt *et al.*, 1995; Chi *et al.*, 1996; Lounsbury *et al.*, 1996; Richards *et al.*, 1996). The GDP-bound form of Ran interacts with NTF2 (Nehrbass and Blobel, 1996; Paschal *et al.*, 1996) as well as with a complex containing RanBP1 and p97 (Chi *et al.*, 1996, 1997). The precise stages in the nuclear transport cycle at which these nucleotide-specific interactions of Ran take place remain controversial (Melchior *et al.*, 1995; Rexach and Blobel, 1995; Görlich *et al.*, 1996).

The initial binding of transport substrate to the NPC during nuclear import is proposed to occur at RanBP2 (Melchior *et al.*, 1995), which is localized at the cytoplasmic fibrils of the NPC (Wilken *et al.*, 1995; Wu *et al.*, 1995; Yokoyama *et al.*, 1995). RanBP2 is a large protein of 358 kDa that contains a number of distinct structural domains including four RanGTP-binding regions, several areas with FG repeat motifs, a zinc-finger domain, a leucine-rich region, and a cyclophilin-like domain (Wu *et al.*, 1995; Yokoyama *et al.*, 1995). However, the potential roles of these various domains of RanBP2 in the nuclear import process have yet to be defined. The FG repeat domains of some other nucleoporins mediate an interaction with p97 in vitro (Iovine *et al.*, 1995; Kraemer *et al.*, 1995; Radu *et al.*, 1995b; Hu *et al.*, 1996) and may serve a similar function in RanBP2. Consistent with this possibility RanBP2 is able to bind to p97 in a blot overlay assay (Moroianu *et al.*, 1995b).

The four RanGTP-binding domains of RanBP2 are 46–60% identical to the RanGTP-binding domain of RanBP1 (Yokoyama *et al.*, 1995). The binding of RanGTP to RanBP2 has not yet been characterized and may be very different from the binding of RanGTP to RanBP1. This is because of the sequence differences between the RanGTP-binding domains of RanBP1 and RanBP2 and because of the large surrounding regions in which the RanGTP-binding domains of RanBP2 are embedded. The presence of both RanGTP-binding domains and FG repeat domains in RanBP2 suggests that it plays a more complex role in nuclear import than the other nucleoporins.

Several lines of evidence indicate that binding and hydrolysis of RanGTP at RanBP2 are required for transport. First, when nuclear import is inhibited by nonhydrolyzable GTP analogues, Ran accumulates at the cytoplasmic fibrils of the NPC, at a site coincident with the localization of RanBP2 (Melchior *et al.*, 1995). In addition, the NPC-bound RanGAP1 is tightly associated with RanBP2, and binding of antibodies to this population of RanGAP1 strongly inhibits nuclear protein import (Mahajan *et al.*, 1997). This inhibition cannot be relieved by the addition of active cytosolic RanGAP1, implying that the localization of RanGAP1 at RanBP2 is central to the function of RanGAP1 in import and that RanGAP1 does not exist solely to generate cytosolic RanGDP (Mahajan *et al.*, 1997). However, the lack of purified RanBP2 has thwarted any detailed characterization of its interaction with nuclear import factors and its putative role in nuclear import.

Here we present a procedure for purifying RanBP2 from rat liver nuclear envelopes. We present structural data suggesting that RanBP2 is the primary component of the cytoplasmic fibrils of the NPC, which may undergo conformational changes to carry the transport substrate from the periphery of the NPC to the central channel. We have biochemically characterized the interaction of RanBP2 with Ran and p97 and demonstrate that RanGTP acts in selectively targeting p97 to RanBP2, where p97 may support the binding of a

substrate/NLS-receptor complex to RanBP2 in an early step of nuclear import at the NPC.

MATERIALS AND METHODS

RanPB2 Purification from Rat Liver Nuclei

RanBP2 was purified from 2000 OD_{280} of salt-washed rat liver nuclear envelopes (NE). NE were solubilized for 2 h in 40 ml Buffer A (20 mM Tris-HCl, pH 8.8, 2% Triton X-100, 1 M NaCl, 2 mM dithiothreitol (DTT), and 5 μg/ml of each of the following protease inhibitors: aprotinin, leupeptin, pepstatin, E64, Pefabloc). Solubilized proteins were diluted 10-fold in buffer B (50 mM HEPES, pH 7.4, 2 mM DTT, protease inhibitors) and centrifuged at 100 000 $\times$ *g* for 1 h. The supernatant was incubated overnight with Ran-coated agarose beads (see below). The beads were washed four times with buffer B plus 0.2% Triton X-100, once with buffer B plus 0.2% Triton X-100 and 0.1% Empigen BB, and once with buffer C (20 mM HEPES, pH 7.4, 110 mM KOAc, 2 mM MgOAc, 1 mM EGTA). Proteins were eluted with buffer C plus 10 mM EDTA, 0.2 M NaCl, and 1% Empigen BB. RanBP2 was further purified by fast protein liquid chromatography (FPLC) using a Mono-Q column in 50 mM HEPES, 1% Empigen BB, 2 mM DTT, and eluted with a 100–700 mM NaCl gradient. Fractions (200 μl) were analyzed by SDS-PAGE and silver staining. Fractions containing RanBP2 were pooled, and aliquots were stored at −70°C. During fractionation, protein concentrations were estimated using the Bio-Rad protein assay (Bio-Rad, Richmond, CA) and Coomassie blue staining of SDS-PAGE using bovine serum albumin (BSA) as a standard. Yield of RanBP2 purification was determined by comparative Western blots.

Preparation of Ran-coated Agarose Beads for RanBP2 Purification

The coding sequence of 86 C-terminal amino acids of the bacterial biotin carboxy carrier protein (BCCP; Chapman-Smith *et al.*, 1994) were amplified by polymerase chain reaction from *Escherichia coli* and fused to the N-terminal end of the Ran cDNA. The construct was introduced into the pGEX-KG vector (Pharmacia LKB, Piscataway, NJ) in which the glutathione S-transferase (GST) coding sequence at the *Eco*N1/*Bam*HI sites has been deleted. BCCP-Ran expression in DH5α was induced at 37°C with 0.1 mM isopropyl-β-D-thiogalactopyranoside for 14 h in Luria-Bertani medium. The cells (500 ml) were then harvested at 6000 $\times$ *g*, 4°C, for 30 min and washed in lysis buffer (50 mM Tris-HCl, pH 8.0, 200 mM NaCl, 1 mM MgCl2, 1 mM EGTA, 2 mM DTT, 1 μg/ml phenylmethylsulfonylfluoride, 2 μg/ml aprotinin, leupeptin, and pepstatin) and recentrifuged. The pellet was then resuspended in 20 ml lysis buffer and frozen at −70°C. The frozen cells were thawed at room temperature and lysed with 1 mg/ml of lysozyme for 1 h at 4°C. Cell debris was pelleted at 100 000 $\times$ *g* for 1 h. Triton X-100 was added to the supernatant at 1% final concentration, and the solution was incubated with 4 ml of streptavidin-agarose beads (Sigma Chemical, St. Louis, MO) for 4 h at 4°C. The beads were then washed four times with 1% Triton X-100 in lysis buffer and twice with 50 mM HEPES, pH 7.4, 2 mM DTT. The bead-bound Ran was loaded with GDP by incubation for 30 min at 30°C with 200 μM GDP in 50 mM HEPES, pH 7.4, 2 mM DTT, 10 mM EDTA. The reaction was stopped at 4°C by addition of 20 mM $MgCl_2$, and the beads were washed in 50 mM HEPES, pH 7.4, 2 mM DTT, 1 mM $MgCl_2$.

Expression of Recombinant Proteins

Recombinant proteins were expressed in *E. coli* BL21 (DE3) and purified as described by Melchior *et al.* (1995) for Ran or as described by Hu *et al.* (1996) for GST-p97, $(His)_6$-p97, and $(His)_6$-SRP1.

Micro Plate Assay

Microtiter plates were coated with 2.5 ng purified RanBP2 or 25 ng GST-p62 per well by incubation for 24 h in coating buffer (phosphate-buffered saline [PBS] plus 4 mM DTT and 2 μg/ml of the protease inhibitors [E64, phenylmethylsulfonylfluoride, apoprotinin, leupeptin, and pepstatin]). After coating, the plates were incubated overnight with binding buffer (3% BSA and 0.1% Tween 20 in coating buffer). Binding reactions involving Ran and/or 6xHis-p97 were carried out for 1 h at room temperature with 100 μl/well of the indicated protein, and the wells were subsequently washed three times with binding buffer without BSA. For analysis of p97 binding, proteins were cross-linked for 15 min with 1 mg/ml EDC (Pierce Chemical, Rockford, IL) in the same buffer. The wells were then washed for 20 min with PBS-T (PBS, 0.2% Tween 20), 10 min with PBS-T plus 100 mM ethanolamine, and 10 min with PBS-T plus 3% BSA. The bound 6xHis-tagged p97 was detected using antiXpress antibody (Invitrogen, San Diego, CA) and horseradish peroxidase-conjugated secondary antibody (Pierce Chemical). Colorimetric detection was carried out using 0.4 mg/ml *O*-phenylenediamine (Sigma Chemical) in 0.0012% H_2O_2, 50 mM Na_2HPO_4, 27 mM Na-citrate, pH 5. The reaction was stopped by addition of 2 M H_2SO_4, and the signal was measured at 490 nm using a microplate reader (Bio-Rad model 3550). For analysis of Ran binding, bound Ran was recovered from the plates with a 2% SDS solution, and the radioactivity was counted in a liquid scintillation counter.

Loading of Ran with GTP or GDP

To study the effect of RanGTP or RanGDP on p97 binding to RanBP2 or p62, 1μM of recombinant Ran (essentially Ran-GDP) was incubated with 1mM of nucleotide in the presence of 10 mM EDTA, 2 mM ATP, 4 mM DTT, 50 mM HEPES, pH 7.4, for 30 min at 30°C. The reaction was stopped at 4°C by addition of 15 mM $MgCl_2$. To study the binding of Ran to RanBP2, Ran was loaded using 6μM of 40 μCi/mmol GTPγ^{32}P or GDPβ^{32}P. Loaded Ran was separated from free nucleotides using a NAP5 column (Pharmacia Biotech, Piscataway, NJ) that had been equilibrated in Buffer C (50 mM HEPES, 2 mM $MgCl_2$, 4 mM DTT, 0.1% BSA, 0.005% Tween 20). For loading of Ran with GTPγ^{32}P, the final RanGTP concentration was adjusted with cold RanGTP.

p97 Blot Overlay

For blot overlay procedures, proteins present in 2 OD_{260} of salt-washed NE were separated on a 5–15% gradient SDS-PAGE and transferred to nitrocellulose membrane. Proteins were denatured in 6 N Guanidine HCl, 50 mM Tris-HCl, pH 7.4, 4 mM DTT and slowly renatured by three rounds of dropwise addition of 10 volumes of 50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 2 mM $MgCl_2$, 2 mM $CaCl_2$, 0.2 mM $ZnCl_2$, 4 mM DTT. The membrane was then blocked with PBS containing 3% BSA plus 4 mM DTT. Binding reactions were carried out with 20 nM GSTp97 in the absence or presence of 40 nM Ran in 1 ml of 0.5% BSA, 0.1% Tween 20 in PBS/4 mM DTT for 30 min at room temperature. After incubation, the membrane was washed in incubation buffer without BSA, and the bound GST-p97 was cross-linked with 5 mM EDC (Pierce). GST-p97 was then detected by using anti-GST antibodies (Pharmacia), peroxidase-conjugated anti-mouse antibodies (Pierce), and ECL reagents (Pierce).

Electron Microscopy and Immunogold Labeling

Electron microscopy of purified RanBP2 utilized negative staining: 2 μl of RanBP2 (at 2.5 μg/ml) were dropped on carbon-coated grids, and the excess solution was blotted on the edge with a piece of filter paper. The grids were immediately washed three times with 15 μl of 2% uranyl acetate and then finally incubated with the 2% uranyl acetate for 1 min. Excess stain was removed, and the grid was air-dried before visualization. For immunogold labeling, isolated rat liver nuclear envelopes (NE) at a concentration of 250 OD_{260}/ml

were incubated for 30 min on ice either with GST-p97 (5 μg/ml) or with GST-p97 premixed with RanGTP (10 μg/ml). After incubation, NE were centrifuged at 6000 × *g* and washed with PBS. p97 bound to NE was labeled by incubation with anti-GST antibody followed by anti-goat IgG conjugated to 5-nm gold particles for 2 h at room temperature. The pellets were fixed with 2% glutaraldehyde in PBS for 30 min at room temperature, washed with PBS, and postfixed with 1% osmium tetroxide in PBS for 1 h. Finally, samples were dehydrated and embedded in Epon 812 resin. Sections were stained with 2% uranyl acetate for 1 min (Guan *et al.*, 1995). All micrographs were recorded with a Hitachi 600 electron microscope at 80 kV.

RESULTS

Purification and Structural Analysis of RanBP2

We established a procedure to purify RanBP2 from salt-washed rat liver NE (Figure 1). The initial preparation of NE from intact rat liver results in an approximate 200-fold enrichment of NPC proteins versus total proteins (Snow *et al.*, 1987; our unpublished observations). NE (Figure 1A, lane 2) were first incubated in a buffer containing Triton X-100 and high salt at pH 8.8 to solubilize RanBP2 (Figure 1A, lane 3). We then used an affinity purification step to take advantage of the four functional Ran-binding domains in RanBP2. A fusion protein containing the biotinylated region of the *E. coli* BCCP was expressed in *E. coli*, and the resultant Ran-BCCP was bound to streptavidin agarose beads. Solubilized NE proteins were incubated with this affinity matrix, and RanBP2 was eluted with a buffer containing the zwitterionic detergent EmpigenBB, which was stringent enough to efficiently elute RanBP2 without removing Ran-BCCP (Figure 1A, lane 5; and our unpublished observations). The eluted RanBP2 fractions were then purified on a MonoQ column (Figure 1A, lane 6). The final pool from the MonoQ contained no contaminants detectable by silver staining (Figure 1B). The yield of RanBP2 from purified rat liver NE with this procedure was ~5%, and about 2.5 μg of RanBP2 was obtained from 2000 OD_{260} of NE (Table 1).

We characterized the structure of purified RanBP2 by electron microscopy using negative staining (Figure 2). We observed two distinct but related structural views of RanBP2, depending on the specific electron microscopic (EM) sample examined. In some EM samples (Figure 2A), most of the particles were filaments with a length of ~36 nm and a diameter of about 5 nm and were usually bent or curved in an irregular manner. In other preparations (Figure 2B), most of the particles existed as spherical structures that appeared to be coiled or spiral conformations of the filamentous particles. A gallery of particles that appeared as spiral filaments is shown in Figure 2C. Each type of sample showed some particles with a structure that was intermediate between the two morphologies, suggesting that RanBP2 forms a flexible filamentous protein that is able to undergo conformational changes, such as coiling (Figure 2, A and B, arrows). Because the same biochemical preparations of purified RanBP2 yielded EM samples that contained either predominantly spiral particles or curved filaments, it appears that the conformation of RanBP2 on EM grids is determined by some aspect of the EM sample preparation (e.g., grid surface charge). Interestingly, the length of purified RanBP2 is very similar to the length of the cytoplasmic fibrils of the NPC that are visualized in freeze-dried, rotary shadowed preparations (35–50 nm, Jarnik and Aebi, 1991), and that also appear to be flexible structures (Panté and Aebi, 199b). Considered together, our

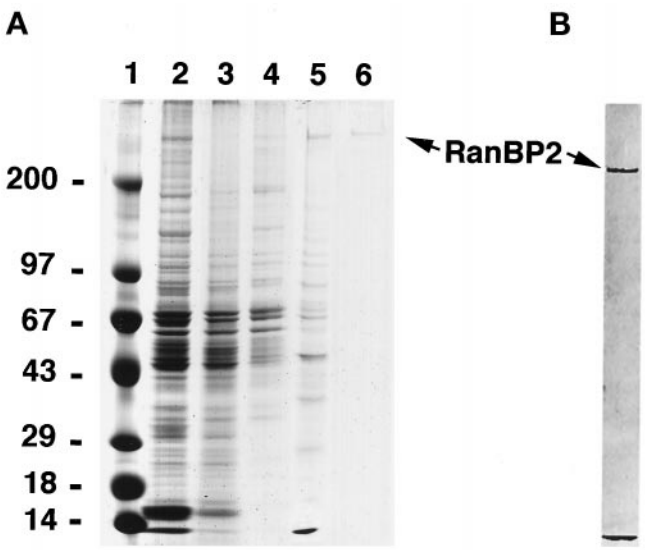


Figure 1. Purification of RanBP2 from rat liver NE. Proteins obtained during different steps of the purification were analyzed by SDS-PAGE on a 5–20% gradient gel followed by staining with Coomassie blue. (A) Lane 1, molecular weight standards (molecular weights indicated to the left of lane 1); lane 2, total NE; lane 3, salt-washed NE; lane 4, solubilized NE proteins; lane 5, proteins eluted from Ran affinity beads; lane 6, purified RanBP2. Proteins in lanes 2–4 were derived from 1 OD_{260} NE, and proteins in lanes 5–6 were derived from 20 OD_{260} NE. (B) Purified RanBP2 (from 20 OD_{260} NE) was analyzed by silver staining after SDS-PAGE as in panel A

Table 1. Purification of RanBP2

Step	Proteins (μg)	RanBP2 (μg)	Fold-purification		Yield (%)
			Step	Cumulative	
Starting material (NE)	22,000	50			100
Salt wash	15,000	48	1.4	1.4	98
Solubilization	10,000	37	1.1	1.6	74
Ran affinity	200	17	23	37	34
Mono Q	2.5	2.5	11.7	440	5

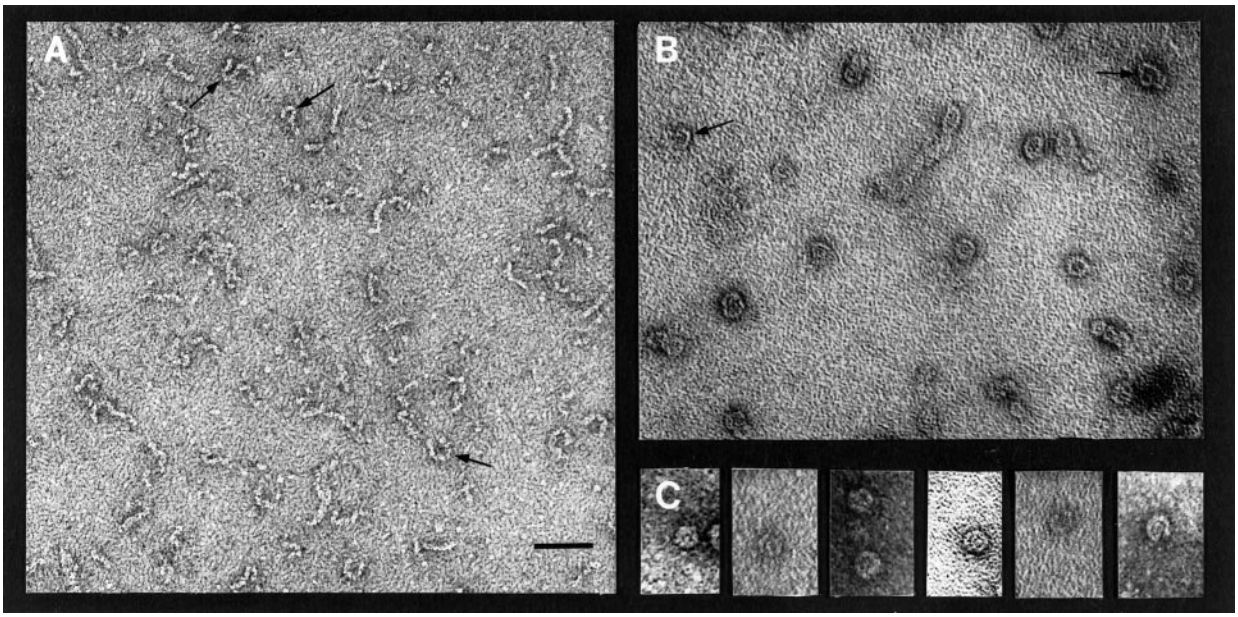


Figure 2. Electron microscopy of negatively stained RanBP2. Purified RanBP2 was adsorbed to glow-discharged carbon-coated grids and was negatively stained with uranyl acetate. Panels A and C are electron micrographs of two different grid preparations that show predominantly an extended (panel A) or folded (panel B) conformation of RanBP2. Arrows indicate examples of partially folded conformations of RanBP2 found in both preparations. Panel C is a gallery of folded RanBP2 molecules selected from the same preparation as panel B. Bar, 100 nm.

data indicate that RanBP2 is a primary component of the cytoplasmic fibrils of the NPC.

RanGTP Restricts the Binding of p97 to RanBP2 at the Cytoplasmic Surface of the NPC

Previous studies have shown that p97 interacts with multiple FG repeat nucleoporins in vitro, supporting the notion that p97 mediates the binding of the transport substrate/NLS receptor complex to multiple regions of the NPC (Moroianu *et al.*, 1995a; Radu *et al.*, 1995a; Hu *et al.*, 1996). Because RanGTP inhibits the formation of a complex between p97 and certain yeast nucleoporin fragments and/or dissociates this complex (Rexach and Blobel, 1995), we investigated whether RanGTP interferes with the binding of p97 to RanBP2 and other nucleoporins of mammalian cells. As shown in the blot overlay assay in Figure 3 (lane 1), p97 by itself binds to numerous nuclear envelope proteins representing FG repeat-containing nucleoporins, as previously reported by Moroianu *et al.* (1995b). The presence of RanGTP in the binding reaction abolished all detectable interaction of p97 with most of these proteins (Figure 3, lane 3), while RanGDP had no effect (Figure 3, lane 2). Interestingly, the presence of RanGTP enhanced the binding of p97 to a ~350-kDa protein that comigrates with RanBP2 (Figure 3, lane 3). We obtained the same result using purified RanBP2 (Figure 3, lanes 4 and 5) confirming the identity of the ~350-kDa band. Thus, under these conditions RanBP2 is the only NPC protein whose interaction with p97 persists (and is enhanced) in the presence of RanGTP.

To assess the effect of RanGTP on the binding of p97 to native NPCs, we carried out p97 binding experiments with isolated rat liver nuclear envelopes and then localized the bound p97 by immunogold EM. As shown in Figure 4, p97 bound to both the nucleoplasmic and cytoplasmic sides of the NPC in the absence of RanGTP (panels A and B). Sixty-eight percent of the gold particles were localized on the cytoplasmic side of the NPC at an average distance of approximatively 50 nm from the NPC midplane, whereas 32% of the gold particles were on the nucleoplasmic side of the NPC at an average distance of about 45 nm from the pore midplane (Figure 4E). In contrast, in the presence of RanGTP, p97 bound almost exclusively (92% of the gold particles) to the cytoplasmic side of the NPC at an average distance of about 50 nm, where RanBP2 is localized (Melchior *et al.*, 1995; Wilken *et al.*, 1995; Wu *et al.*, 1995; Yokoyama *et al.*, 1995). These results are consistent with the demonstration that the presence of RanGTP restricts binding of p97 to RanBP2 in blot overlay assay (Figure 3) and support the notion that RanGTP targets p97 to RanBP2 in the context of the native NPC structure.

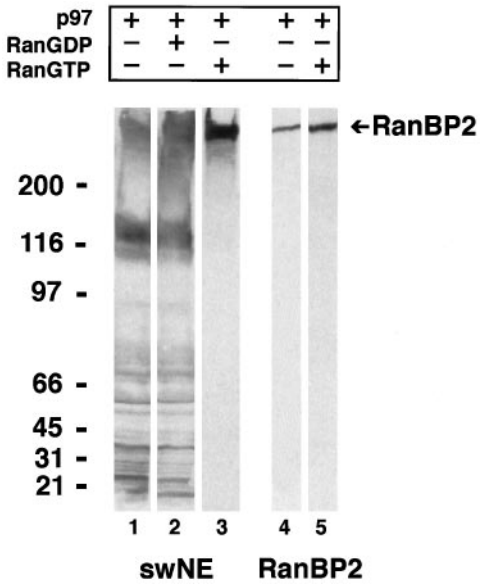


Figure 3. p97 binds selectively to RanBP2 in the presence of RanGTP. A blot overlay approach was used to analyze the binding of p97 to proteins of salt-washed NE (swNE, 2 OD_{260}, lanes 1–3), or to purified RanBP2 (10 ng, lanes 4–5). Protein samples were separated by SDS-PAGE on a 5–15% gradient gel, electrophoretically transferred to nitrocellulose, and the nitrocellulose membrane was renatured. Nitrocellulose strips were incubated with GSTp97, RanGDP, and RanGTP as indicated. After cross-linking, bound p97 was detected using anti-GST antibodies.

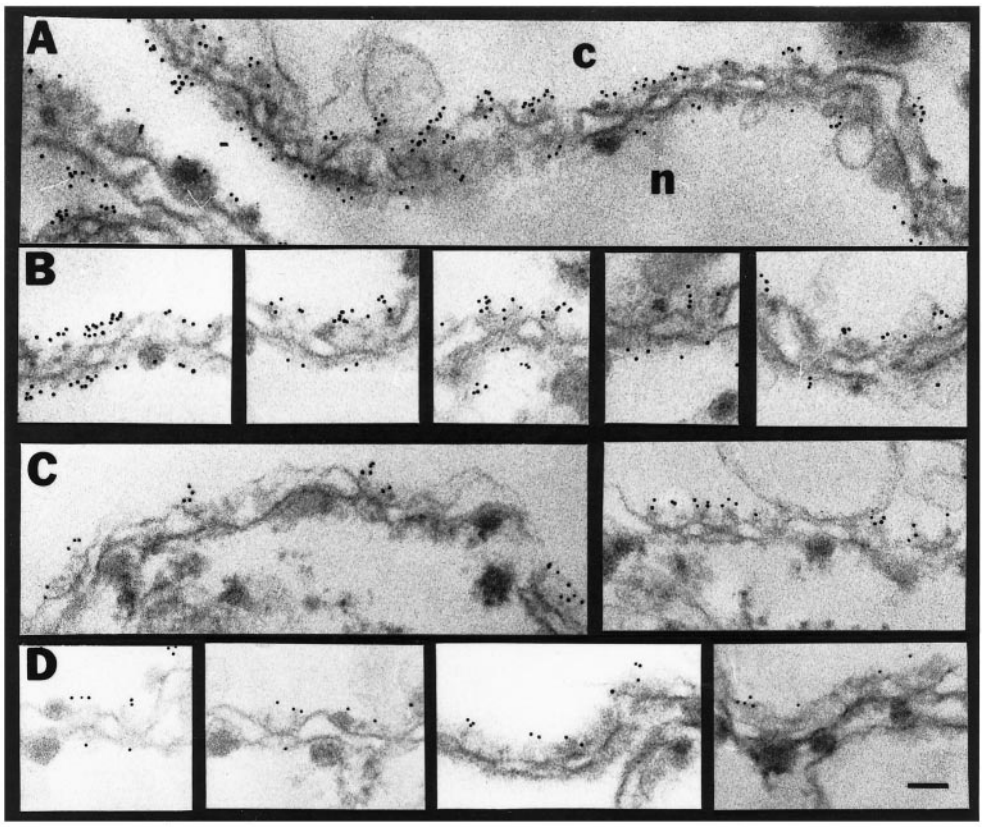
Characterization of the Binding of p97 and Ran to RanBP2

To investigate the molecular basis for the binding of p97 to RanBP2 in the presence of RanGTP, we quantitatively analyzed the binding of p97 and RanGTP to RanBP2 using a microtiter plate-binding assay. Figure 5A shows that the interaction of RanGTP with purified RanBP2 is saturable and of high apparent affinity (K_{dapp} = 0.5 nM). Because there are four distinct Ran-binding sites in RanBP2, this must be regarded as an average affinity. This affinity is significantly higher than that of RanGTP for RanBP1 under the same conditions (K_{dapp} = 1.4 nM; our unpublished results), and may either be caused by sequence differences between the RanBP1 and RanBP2 Ran binding domains, by the presence of adjacent domains in RanBP2 that are not found in RanBP1 or by the presence of four Ran binding sites in RanBP2 as opposed to one in RanBP1, which could lead to a higher local concentration of Ran (Wu *et al*., 1995; Yokoyama *et al*., 1995). RanGDP did not bind significantly to RanBP2 (Figure 5A) or to RanBP1 (our unpublished results) under these conditions. We also used the microtiter plate-binding assay to compare the binding affinity of p97 for RanBP2 and for p62, a nucleoporin that does not bind Ran but that also contains FG repeats (Starr *et al*., 1990). We found that p97 bound both to RanBP2 (Figure 5B) and to p62 (Figure 5C) with an apparent K_d of 14 nM. Thus, RanGTP binds to RanBP2 with an apparent affinity that is nearly 30 times higher than the affinity of p97 for RanBP2 or p62.

We next investigated the effect of Ran on the binding of p97 to RanBP2 and p62 under the above conditions. Figure 6 shows that a low concentration of RanGTP stimulated the interaction of p97 with RanBP2 by approximately 50% (panel A) but had no stimulatory effect on the binding of p97 to p62 (panel B). As the RanGTP concentration was further increased, the extent of interaction of p97 with both RanBP2 and p62 decreased and was eventually abolished, although the binding of p97 to p62 was more strongly inhibited at a given concentration of RanGTP than was the binding of p97 to RanBP2. RanGDP had no significant effect on the binding of p97 to RanBP2 and p62.

A simple interpretation of these data is that Ran-binding sites and FG-repeat regions comprise two different classes of binding sites for p97, and that Ran differentially affects the binding of p97 to these two classes of sites. In the case of the FG-repeat regions, free p97 could bind to these regions in both RanBP2 and p62, and RanGTP could inhibit this binding through the formation of a RanGTP/p97 complex. This was observed previously for yeast nucleoporin fragments (Rexach and Blobel, 1995). In the case of the Ran-binding sites, RanGTP could stimulate the binding of p97 to RanBP2 by forming a RanGTP/p97 complex. Because the Ran-binding motifs of RanBP2 are similar to those of RanBP1 and because previous experiments have shown that a stable heterotrimeric complex can form between RanGTP, p97, and RanBP1 (Chi *et al*., 1996; Lounsbury *et al*., 1996), it is plausible that a similar trimeric complex could form between RanGTP, p97, and RanBP2. However, at high RanGTP concentrations, excess RanGTP would compete with the RanGTP/p97 complex for binding to the Ran-binding sites on RanBP2.

To directly test whether a complex between p97 and RanGTP is important for regulating the binding interactions of p97 with RanBP2 and p62, we analyzed a p97 mutant deficient in Ran binding (Görlich *et al*., 1996) for its ability to interact with RanBP2 and p62 in the absence and presence of RanGTP. The Δ54N-p97 used in these experiments was slightly shorter that the Δ44N-p97 mutant characterized by Görlich and coworkers (1996). We confirmed that RanGTP did not bind to the Δ54N-p97 mutant (Figure 7A). In the absence of RanGTP, the binding of Δ54N-p97 to both RanBP2 and p62 was similar to that of the wild-type (wt) p97 (Figure 7, B and C). However, in contrast to wt p97, the binding of Δ54N-p97 to RanBP2 was not stimulated by low concentrations of RanGTP, and the binding of Δ54N-p97 to either RanBP2 or p62 was not diminished by higher concentrations of RanGTP (Figure 7, B and C). These results clearly indicate that



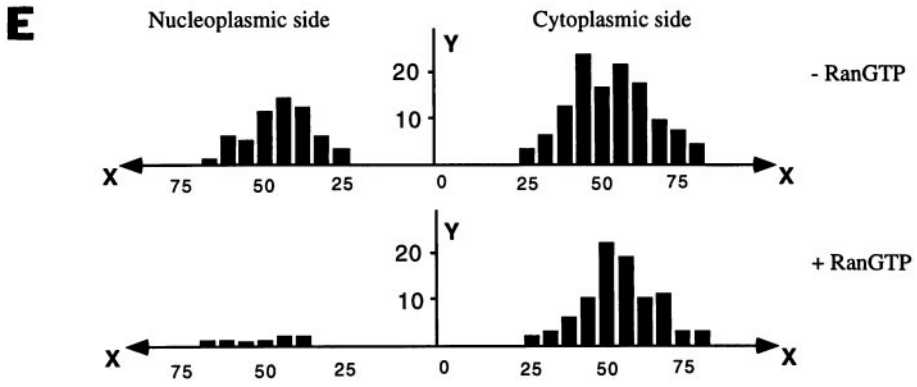


Figure 4. RanGTP selectively targets p97 to the cytoplasmic side of the NPC. GST-p97 was incubated with isolated rat liver NE in the absence (A and B) or presence (C and D) of RanGTP. Bound p97 was visualized by labeling with goat anti-GST antibodies followed by rabbit anti-goat antibodies coupled with 5 nm gold. Samples were then processed for thin section electron microscopy. (E) Histograms showing the distance of gold particles from the midplane of the NPC for the binding of p97 in the absence (top) or presence (bottom) of RanGTP. X axis: distance of gold particles from the midplane; Y axis: number of gold particles counted; c, cytoplasm; n, nucleoplasm.

RanGTP regulates the binding of p97 to RanBP2 and p62 through formation of a complex with p97.

DISCUSSION

We have developed a procedure for the purification of RanBP2 from rat liver NE under relatively mild conditions. Electron microscopy of negatively stained RanBP2 revealed it to be a flexible filamentous protein capable of adopting a tightly coiled spiral conformation. RanBP2 has previously been localized to the cytoplasmic NPC fibrils, which appear to be flexible structures (Panté and Aebi, 1996b) with a length of 35–50 nm in freeze-dried, rotary-shadowed preparations (Jarnik and Aebi, 1991; Goldberg and Allen, 1993). Purified RanBP2, after negative staining, had a length of about 36 nm. Thus, the localization, size, and flexibility of RanBP2 are similar to the properties of the cytoplasmic fibrils of the NPC. In addition, RanBP2 in its coiled conformation quite closely resembles the granules observed at the periphery of isolated

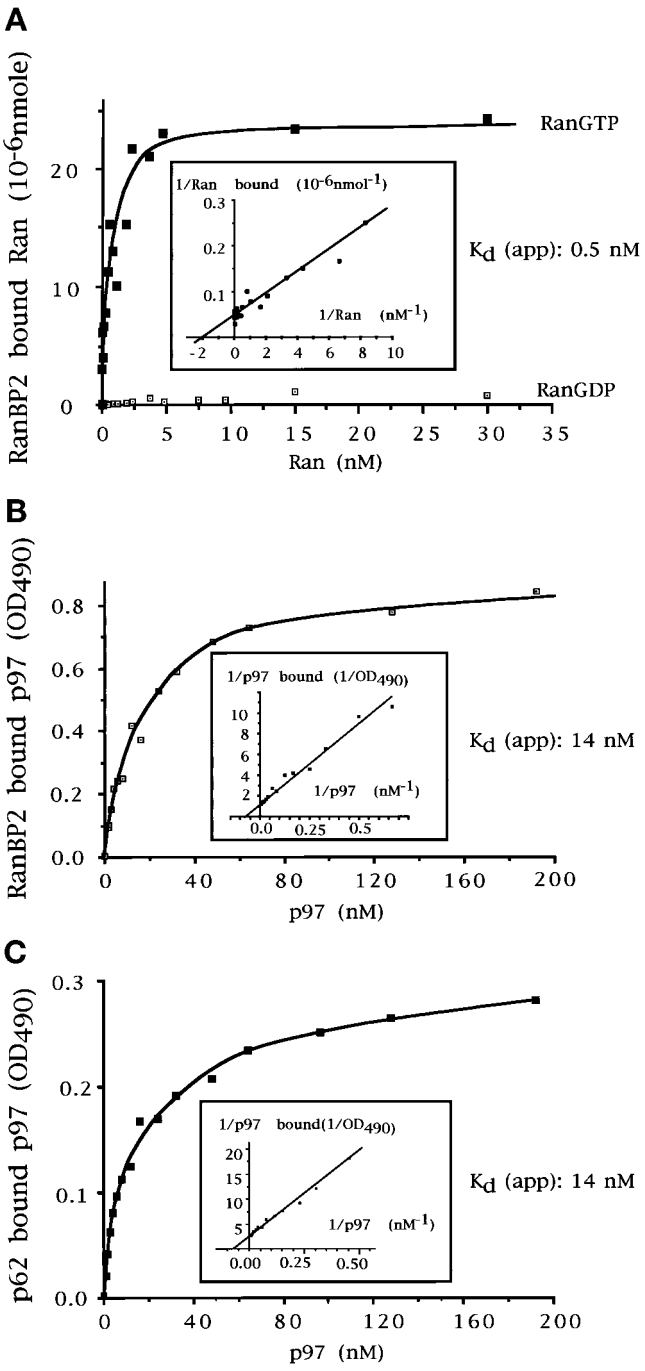


Figure 5. Characterization of the interactions of Ran and p97 with nucleoporins RanBP2 and p62. (A) Titration of Ran binding to RanBP2. Increasing concentrations of Ran loaded with either [^{32}P]GTP or [^{32}P]GDP were incubated with RanBP2 adsorbed to microtiter wells. After washing, the radioactivity was recovered from the wells with SDS and counted in a liquid scintillation counter, and the amount of Ran bound to RanBP2 was calculated from the [^{32}P]GTP-specific activity. (B and C) Titration of p97 binding to RanBP2 and p62, respectively. Increasing concentration of His-p97 were incubated with RanBP2 (B) or p62 (C) and adsorbed to microtiter wells. The 6xHis-p97 that remained after washing was then cross-linked to the adsorbed protein and detected with antibodies (see MATERIALS AND METHODS). The Lineweaver-Burk plots used to determine the apparent binding constants ($K_{dapp.}$) are shown in the insets.

NPCs (Unwin and Milligan, 1982; Stewart and Whytock, 1988; Goldberg and Allen, 1993), which may represent a compacted/collapsed form of the cytoplasmic fibrils as suggested by Jarnik and Aebi (1991). Considered together, these observations suggest that RanBP2 may form the backbone of the cytoplasmic fibrils of the NPC.

One model for the movement of a transport complex from an initial docking side at the cytoplasmic fibrils of the NPC to central pore regions proposes the involvement of fibril bending (Melchior and Gerace, 1995; Panté and Aebi, 1995). This hypothesis has gained support from a recent electron microscopy study analyzing the movement of gold-coupled substrate through the NPC of *Xenopus* oocytes (Panté and Aebi, 1996b). Our present data lend further credence to this hypothesis, as indicated by the ability of RanBP2 to adopt an extended or bent/coiled conformation in vitro. The process leading to these conformational changes may be unregulated (as proposed by Panté and Aebi, 1996b), or could be controlled in the native NPC by interactions with cytosolic import factors such as p97 and Ran. Future analysis of conformational changes of RanBP2 in vitro should provide valuable mechanistic insight on the movement of an import complex from the periphery of the NPC to the central channel.

Using blot overlay and microtiter plate binding assays, we demonstrated that RanGTP at appropriate concentrations both promotes the binding of p97 to RanBP2 and inhibits the binding of p97 to p62 and to other FG repeat nucleoporins. In agreement with these data, EM analysis showed that RanGTP can restrict the binding of p97 to the cytoplasmic periphery of the NPC, where RanBP2 is found. Using a p97 mutant unable to bind RanGTP, we found that the ability of RanGTP to modulate the binding of p97 to RanBP2 and p62 is dependent upon the formation of a RanGTP/p97 complex. In agreement with these results, the ability of anti-RanBP2 antibodies to coimunoprecipitate p97 and Ran from a *Xenopus* egg extract is nucleotide dependent (Saitoh *et al*., 1996).

Our data suggest that two different classes of binding sites for p97 are responsible for these effects. First, the FG repeat domains present in both RanBP2 and p62 appear to be able to bind to p97 alone, each with an apparent K_d of about 14 nM. The presence of RanGTP, which promotes formation of a RanGTP/p97 complex, appears to inhibit the binding of p97 to this class of sites on both RanBP2 and p62. These results are in agreement with a previous study on the binding

of p97 to fragments of yeast nucleoporins containing FG repeats (Rexach and Blobel, 1995). In addition, RanBP2 contains specific binding sites for RanGTP (K_{dapp} = ~0.5 nM) that are absent from p62 and from other FG repeat nucleoporins. These sites on RanBP2 are homologous to those of RanBP1 (Wu *et al.*, 1995; Yokoyama *et al.*, 1995) and appear to be capable of binding p97 in the form of a RanGTP/p97 complex as was previously described for RanBP1 (Chi *et al.*, 1996; Lounsbury *et al.*, 1996). Thus, in the case of this class of p97-binding sites, the presence of RanGTP leads to formation of a RanGTP/p97 complex and has a positive effect in promoting the binding of p97 to RanBP2. Because this second class of binding sites for p97 is present in RanBP2 but in no other FG repeat nucleoporins, appropriate concentrations of RanGTP can suppress the binding of p97 to all nucleoporins except RanBP2, to which the binding is enhanced.

In vivo, a complex of RanGTP and p97 could be formed in the late stages of a nuclear import cycle and exported to the cytoplasm from the nucleoplasm (see Görlich *et al.*, 1996) or could be created in the cytoplasm after RanGTP exits the nucleus in another form. Our in vitro binding experiments indicate that the RanGTP/p97 complex would be selectively targeted to RanBP2 via its RanGTP-binding sites and would not interact with FG repeat nucleoporins in other regions of the NPC. We propose that in vivo, the p97 bound to RanBP2 in this manner provides a docking site for a transport substrate/NLS receptor complex that is initially formed in the cytosol. This would explain how the initial binding of transport substrates to the NPC is restricted to the cytoplasmic fibrils, as indicated by previous work (Newmeyer and Forbes, 1988; Richardson *et al.*, 1988; Panté and Aebi, 1996b).

The hydrolysis of RanGTP during nuclear import very likely occurs at RanBP2 due to the action of RanGAP1 bound to this protein (Mahajan *et al.*, 1997) and could have two different functions in the proposed substrate/NLS receptor docking reaction at RanBP2. In one case, the GTP would be hydrolyzed upon binding of RanGTP/p97 to RanBP2 and, in so doing, would provide a binding site consisting of RanGDP, p97, and RanBP2 for the substrate/receptor complex at the NPC. In a second case, p97 would suppress hydrolysis of RanGTP that is bound to RanBP2, similar to the inhibition of RanGTP hydrolysis by p97 in solution (Floer and Blobel, 1996). In this scenario, RanGTP hydrolysis would be triggered by binding of the substrate/NLS receptor complex to RanBP2-associated p97. In either of these cases, RanGTP hydrolysis would allow formation of a "committed" transport complex at RanBP2. It is plausible that the resulting RanGDP would remain stably associated with the substrate/NLS receptor/p97 transport complex after GTP hydrolysis. This association could "mark" the transport complex as having passed through an initial commitment step and facilitate its movement to downstream binding sites in the NPC, possibly by the ability of RanGDP to interact with NTF2 (Nehrbass and Blobel, 1996; Paschal *et al.*, 1996) or with other factors.

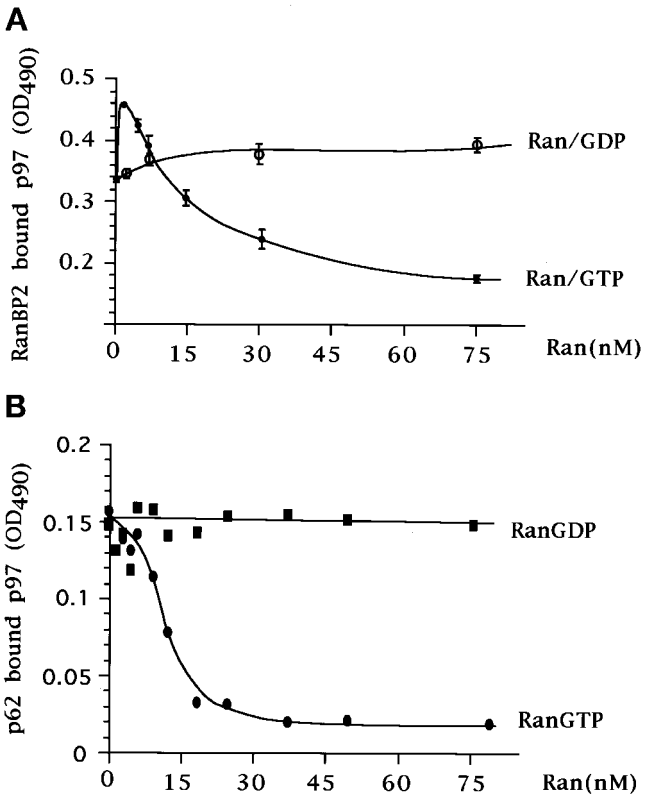


Figure 6. RanGTP modulates the interaction of p97 with RanBP2 and p62. (A) Effect of Ran on the binding of p97 to RanBP2. RanBP2-coated microtiter wells were incubated with 10 nM His-p97 and with an increasing amount of RanGTP or RanGDP. After incubation and washing, proteins were cross-linked, and the amount of bound p97 was quantified using antibodies (see MATERIALS AND METHODS). (B) Effect of Ran on the binding of p97 to p62. The analysis was carried out as in panel A, except that p62-coated microtiter wells were used.

Whatever the precise function of RanGTP hydrolysis, one key feature of the above models is that a complex between the substrate/NLS receptor and p97 first forms at the NPC at RanBP2, and not in the cytosol as was previously proposed by Imamoto and coworkers (1995). Although complexes between the NLS receptor and p97 can be detected in a soluble fraction of cell lysates (Görlich *et al.*, 1995a; Imamoto *et al.*, 1995), this could be attributed to the a conversion of RanGTP to RanGDP in the cell extract. Hydrolysis of RanGTP during the in vitro incubation could convert an initial p97/RanGTP complex (which is incapable of binding the substrate/NLS receptor complex [Rexach and Blobel, 1995]) to a form that can interact with the substrate/NLS receptor. At present, there is

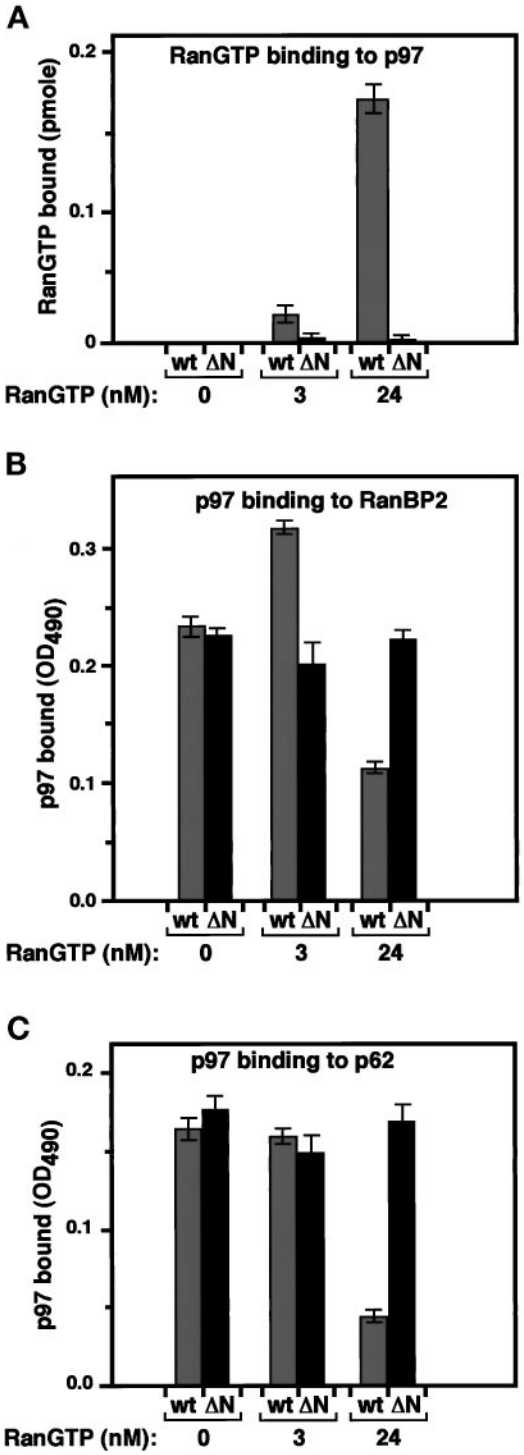


Figure 7. The ability of RanGTP to modulate the binding of p97 to RanBP2 and p62 requires the RanGTP/p97 interaction. (A) 3 nM or 24 nM [^{32}P]GTP-Ran was incubated with wt p97 or ΔN p97 bound to microtiter wells. After binding and washing, the radioactivity was recovered from the plate by elution with SDS and was counted in a liquid scintillation counter. The amount of bound RanGTP was calculated from the [^{32}P]RanGTP-specific activity. (B) The binding of wt p97 and ΔN p97 to RanBP2 adsorbed to microtiter wells was analyzed in the absence of Ran, or in the presence of 3 nM or 24 nM RanGTP, and the bound p97 was quantified as in Figure 4. (C) The binding of wt p97 and ΔN p97 to p62 adsorbed to microtiter wells was analyzed as in panel B.

no clear evidence that cytosolic complexes of NLS receptor/p97 occur in living cells. A second key feature of these models is that RanGTP, in the form of a RanGTP/p97 complex, is required at the cytoplasmic side of the NPC for transport. This predicts that a high concentration of free RanGTP would inhibit import, as has been shown previously (Görlich *et al*., 1996), since RanGTP would compete with a RanGTP/p97 complex for binding to RanBP2.

In conclusion, the data we present here suggest a specific mechanism by which RanGTP could mediate the formation of an import complex at RanBP2 in an early step of nuclear import. This is consistent with previous proposals that RanGTP hydrolysis at RanBP2 is important for defining the vectoriality of nuclear protein import involving basic-type NLS substrates (Melchior *et al*., 1995; Mahajan *et al*., 1997).

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