RanBP1 Stabilizes the Interaction of Ran with p97 in Nuclear Protein Import

Neil C. Chi, Ermoné J.H. Adam, Gina D. Visser, and Stephen A. Adam

Department of Cell and Molecular Biology, Northwestern University Medical School, Chicago, Illinois 60611

Abstract. Three factors have been identified that reconstitute nuclear protein import in a permeabilized cell assay: the NLS receptor, p97, and Ran/TC4. Ran/TC4, in turn, interacts with a number of proteins that are involved in the regulation of GTP hydrolysis or are components of the nuclear pore. Two Ran-binding proteins, RanBP1 and RanBP2, form discrete complexes with p97 as demonstrated by immunoadsorption from HeLa cell extracts fractionated by gel filtration chromatography. A >400-kD complex contains p97, Ran, and RanBP2. Another complex of 150–300 kD was comprised of p97, Ran, and RanBP1. This second trimeric complex could be reconstituted from recombinant proteins. In solution binding assays, Ran-GTP bound p97 with high affinity, but the binding of Ran-GDP to p97 was undetectable. The addition of RanBP1 with Ran-GDP or Ran-GTP increased the affinity of both forms of Ran for p97 to the same level. Binding of Ran-GTP to p97 dissociated p97 from immobilized NLS receptor while the Ran-GDP/RanBP1/p97 complex did not dissociate from the receptor. In a digitonin-permeabilized cell docking assay, RanBP1 stabilizes the receptor complex against temperature-dependent release from the pore. When added to an import assay with recombinant NLS receptor, p97 and Ran-GDP, RanBP1 significantly stimulates transport. These results suggest that RanBP1 promotes both the docking and translocation steps in nuclear protein import by stabilizing the interaction of Ran-GDP with p97.

PROTEINS targeted to the nucleus cross the nuclear envelope through a large proteinaceous supramolecular structure called the nuclear pore complex (NPC)[1] (Feldherr, 1984; reviewed by Newmeyer, 1993; Melchior and Gerace, 1995). The pore complex forms an aqueous channel between the nucleoplasm and cytoplasm allowing diffusion of small molecules <9 nm in diameter, but restricting the movement of larger macromolecules in both directions. Multiple transport mechanisms are thought to exist to transport diverse macromolecules from small proteins to large ribonucleoproteins (Görlich and Mattaj, 1996).

The import of nuclear proteins is mediated by single or bipartite stretches of primarily basic amino acid residues called nuclear localization sequences (NLSs) (Dingwall and Laskey, 1991). One of the earliest steps in nuclear protein import is recognition of the NLS by one of a family of cytoplasmic NLS-binding proteins of 54–56 kD known as the NLS receptor/importin α/karyopherin α (Adam and Gerace, 1991; Görlich et al., 1994; Weis et al., 1995). The NLS-containing proteins are then bound to the nuclear pore via a receptor complex containing the NLS receptor and a second factor, p97/importin β/karyopherin β (Adam and Adam, 1994; Görlich et al., 1995*b*; Moroianu et al., 1995*a*). It is thought that p97 mediates interaction with the pore by dimerization with the NLS receptor and direct association with a subset of a peptide repeat-containing family of nuclear pore complex proteins (nucleoporins) (Iovine et al., 1995; Moroianu et al., 1995*b*; Radu et al., 1995*b*). Interaction of the receptor with an NLS-containing protein is not required for translocation of the NLS receptor and p97 as a small region of the receptor that binds p97, the importin β-binding domain (IBB), can direct nuclear accumulation of a chimeric protein (Görlich et al., 1996; Weis et al., 1996). Subsequent release of the bound receptor complex and translocation through the nuclear pore complex requires the small GTPase Ran/TC4 and GTP hydrolysis (Melchior et al., 1993; Moore and Blobel, 1993). The translocation step in permeabilized cells is enhanced by the addition of another factor that interacts with Ran/TC4, p10/NTF2 (Moore and Blobel, 1994; Paschal and Gerace, 1995).

Ran/TC4, like other GTPases, is thought to act as a molecular switch (Bourne, 1990). The GTPase activity of Ran/TC4 is regulated by two proteins: the Ran nucleotide exchange factor, RCC1 (Bischoff and Ponstingl, 1991*a*,*b*), and the Ran GTPase-activating protein, RanGAP1 (Bis-

Address all correspondence to S.A. Adam, Department of Cell and Molecular Biology, W129, Northwestern University Medical School, 303 East Chicago Avenue, Chicago, IL 60611. Tel.: (312) 503-7525. Fax: (312) 503-7912.

1. *Abbreviations used in this paper*: NLS, nuclear localization sequence; NPC, nuclear pore complex.

choff et al., 1994; Becker et al., 1995). Mutational analysis of RCC1 and RanGAP1 has implicated both factors in protein import and RNA export (for review see Tartakoff and Schneiter, 1995; Corbett et al., 1995), although a direct linkage has not been shown and neither factor is required for import in permeabilized cells.

A family of Ran-binding proteins containing conserved domains for interaction with Ran-GTP has been identified and includes RanBP1, RanBP2 (NUP358), Nup2p, and *C. elegans* F59A2.1 protein (Lounsbury et al., 1994; Beddow et al., 1995; Dingwall et al., 1995; Hartmann and Görlich, 1995). Direct interactions between Ran-GTP and RanBP2 (Melchior et al., 1995*a*; Wu et al., 1995; Yokoyama et al., 1995), a XFXFG repeat-containing nucleoporin, and between Ran-GTP and RanBP1 (Coutavas et al., 1993; Lounsbury et al., 1994) have been demonstrated by protein overlay blot. Because of its peripheral cytoplasmic localization on the NPC and its ability to interact with Ran-GTP and p97, RanBP2 may be the initial docking site for nuclear protein import, thus committing the receptor complex for translocation across the nuclear pore complex (Melchior et al., 1995*a*). RanBP1 is involved in the coactivation of RanGTPase via an interaction with RanGAP1 and Ran-GTP (Ren et al., 1995; Bischoff et al., 1995; Richards et al., 1995) and inhibits GTP dissociation from Ran-GTP (Bischoff et al., 1995). Furthermore, RanBP1 can form a complex with nucleotide-free Ran/TC4 and the guanine nucleotide exchange factor, RCC1 (Bischoff et al., 1995; Hayashi et al., 1995; Saitoh and Dasso, 1995). Ran deletion mutants with the COOH-terminal DEDDDL sequence removed are unable to bind RanBP1 and are defective in RNA export, yet have no apparent effect on protein import (Ren et al., 1995; Richards et al., 1995). However, mutants in a yeast homologue of RanBP1, Yrb1p, were found to be defective in both nuclear import and export (Schlenstedt et al., 1995*b*).

We have investigated the role of the Ran-binding protein RanBP1 in nuclear protein import at the level of physical interactions with the nuclear transport factors p97 and Ran. Docking and transport experiments in digitonin permeabilized cells provide evidence that RanBP1 plays a role in the stabilization of a receptor complex during translocation through the pore by increasing the affinity of Ran-GDP for p97.

Materials and Methods

Cell Culture

HeLa JW36 cells were grown in high glucose DMEM containing 10% neonate bovine serum (Biocell Laboratories, Rancho Dominguez, CA) and penicillin/streptomycin (Gibco Laboratories, Gaithersburg, MD). Cultures were maintained in a humidified incubator at 37°C with 5% CO_2 atmosphere. Cells were trypsinized from plastic culture dishes and reseeded onto 18 × 18 mm glass coverslips at 2 × 10^6 cells/6-well plate 18–24 h before use. Replated cells were stimulated with fresh medium 1–3 h before performing transport assays (Adam et al., 1990; Adam and Adam, 1994). HeLa S3 cells were grown in suspension culture in Minimum Essential Medium modified for suspension cultures containing 10% neonate bovine serum and penicillin/streptomycin.

Expression and Purification of Recombinant Nuclear Transport Factors

Human p97 used for binding and transport assays was expressed and purified as described (Chi et al., 1995). Human RanBP1 was amplified by PCR from a HeLa cDNA library (Clontech, Palo Alto, CA) using the following primers: 5′-CCATGGCGGCCGCCAAGGAC-3′ and 5′-CGACCTCGAGTTATTGCTTCTCCTCAGC-3′. The amplified product was subcloned into the pGEX 4T-1 GST-fusion vector (Pharmacia Biotech Inc., Piscataway, NJ) and transformed into JM109 cells. Cultures were grown to an OD_{600} of 0.7 and induced with 0.5 mM IPTG for 3 h at 37°C. Cells were collected by centrifugation at 5,000 *g* for 10 min and resuspended in 0.02 vol of import buffer containing 2 mM DTT. Soluble RanBP1 was obtained by two cycles of freeze-thaw in the presence of 10 μg/ml pancreatic DNase I (Boehringer Mannheim, Indianapolis, IN). Insoluble material was removed by centrifugation at 100,000 *g* for 30 min. Soluble RanBP1-GST was bound to glutathione agarose beads (Sigma Chem. Co., St. Louis, MO), and cleaved with 20 u of thrombin for 6 h at 22°C. Cleaved RanBP1 was further purified by FPLC MonoQ (Pharmacia). Fractions containing RanBP1 were collected and dialyzed against import buffer containing 2 mM DTT, 0.1 mM PMSF, and 1 μg/ml each of aprotonin, leupeptin, and pepstatin A.

To make recombinant NLS receptor, the mouse pendulin cDNA was subcloned into the pET30a His/S-tag fusion expression vector (Novagen, Madison, WI) and transformed into BL21(DE3) cells. 250-ml cultures were grown at 37°C to an OD_{600} of 0.6 and induced with 1 mM IPTG for 3 h at 30°C. Cells were collected by centrifugation and resuspended in 20 mM Hepes (pH 7.0), 0.5 M NaCl, 1 μg/ml each of aprotonin, leupeptin, pepstatin A, 2 mM DTT and 500 μg of lysozyme and incubated on ice for 30 min. Cells were disrupted by sonication and insoluble material was removed by centrifugation at 100,000 *g* for 30 min. Phenyl sepharose equilibrated in import buffer containing 2 mM DTT and 1 μg/ml each of aprotonin, leupeptin, and pepstatin A, was added to the soluble material and mixed at 4°C for 1 hour. The sepharose was washed two times in import buffer and the bound proteins were eluted with 75% ethylene glycol. Eluted NLS receptor was dialyzed against import buffer containing 2 mM DTT and 1 μg/ml each of aprotonin, leupeptin, and pepstatin A.

p97 used for adsorption experiments was subcloned into pET30a and expressed in BL21(DE3) cells. Cultures were grown at 37°C to an OD_{600} of 0.8 and induced with 1 mM IPTG in the presence of 1 mM $ZnCl_2$ for 3 h at 37°C. Cells were collected by centrifugation and resuspended in 0.025 vol of 20 mM Hepes (pH 7.4), 0.5 M NaCl, 1 μg/ml each aprotonin, leupeptin, and pepstatin A, 2 mM DTT and 40 μg/ml of lysozyme and incubated on ice for 30 min. Cells were disrupted by sonication and insoluble material was removed by centrifugation at 100,000 *g* for 30 min. Further purification was as described (Chi et al., 1995).

Recombinant Ran Protein Purification and Nucleotide Loading

Expression and purification of recombinant human Ran/TC4 was modified from Melchior et al. (1995*b*). Recombinant Ran subcloned into a pET11d vector was transformed in BL21(DE3). Cells were grown to an OD_{600} of 0.8 and induced with 0.9 mM IPTG for 2.5–3 h before harvesting by centrifugation at 6,000 *g*. The cell pellets were frozen overnight at −20°C and thawed at 23°C. Cells were immediately placed on ice and resuspended into 0.025 vol of B1 buffer (50 mM, Tris/HCl, pH 8.0, 75 mM NaCl, 1 mM $MgCl_2$, 2 mM DTT, 1 μg/ml each of aprotonin, leupeptin, and pepstatin A). Lysozyme was added to 1 mg/ml and the suspension was incubated for 90 min on ice. Insoluble material was removed by centrifugation at 100,000 *g* and the supernatant was applied to a 20-ml DEAE Sepharose FF column (Pharmacia) previously equilibrated with five column volumes of B1 buffer. The flow through and the first 20 ml of wash with B1 was collected. The protein was then concentrated by precipitation at 55% ammonium sulfate and stored on ice.

20 μM Ran in Ran loading buffer (50 mM Hepes pH 7.4, 10 mM EDTA, 2.5 mM DTT, and 1 μg/ml each of aprotonin, leupeptin, and pepstatin A) was incubated with 1 mM ATP and 1 mM GDP or GTP at room temperature for 30 min, and then on ice for 15 min. Ran samples were diluted 2.5-fold with import buffer containing 2 mM DTT and 0.1 mM PMSF, and magnesium acetate was added to 10 mM. After incubation on ice for 30 min, Ran was purified by gel filtration on a Superose 12 FPLC column (Pharmacia) to remove free nucleotides. Samples were immediately aliquoted and stored at −80°C.

In Vitro Nuclear Import and Nuclear Pore Binding Reactions

HeLa cells grown on glass coverslips were permeabilized with digitonin as described (Adam et al., 1990; Adam and Adam, 1994). Allophycocyanin

chemically conjugated to a SV40 large T antigen NLS-peptide (APC-NLS) at a ratio of five peptides to each APC was used as a fluorescent reporter in import and binding experiments (Adam et al., 1990). Import experiments were performed for 30 min at 25°C in a 50-μl vol containing 1 μM Ran-GDP, 400 nM p97, 400 nM NLS receptor, 1 μg APC-NLS, 1 mM ATP and 1 mM GTP in import buffer (20 mM Hepes, pH 7.35, 110 mM potassium acetate, 2 mM magnesium acetate, 0.1 mM EGTA, and 2 mM DTT) (Adam and Gerace, 1991). RanBP1 was added in concentrations as indicated. Binding experiments were carried out in a 50-μl vol containing p97 and NLS receptor at a 1:2 molar ratio with 1 μg APC-NLS in import buffer at 4°C or with 1 μg APC-NLS, 1 mM ATP and 1 mM GTP in import buffer at 25°C for 20 min. RanBP1 was added in the proportions indicated to the binding mix with p97 and NLS receptor. The addition of nucleotides in the 4°C incubation had no effect on binding. When nucleotides were not included in the 25°C incubation, the extent of decrease in binding was more heterogeneous between cells, with ~20% of the cells showing somewhat less than a 40% decrease in nuclear envelope binding. We have calculated the concentration of p97 and NLS receptor in HeLa cells at 100–500 nM, based on quantitative immunoadsorptions (data not shown). The concentration of Ran in HeLa cells is at least 10-fold greater.

Accumulation of the APC-NLS on the nuclear envelope or within the nucleus was observed by epifluorescence illumination with a Zeiss Axioskop microscope equipped with a 63× 1.25NA oil immersion objective. Quantitation of fluorescence was performed by analyzing images captured with a CCD camera (Electrim Corp., Princeton, NJ), and relative intensity values were determined and averaged with a computer program written by Dr. Guenter Albrect-Buehler (Northwestern University, Chicago, IL). For binding experiments, the rim of the nucleus as observed at the equatorial plane was measured and for import experiments, the average intensity over the entire nucleus was measured.

Metabolic Labeling and Preparation of RIPA Soluble Fractions

HeLa S3 cells grown in suspension were labeled with 20 μCi/ml L-[^{35}S] Pro-Mix (Amersham, Arlington Heights, IL) in 10% Met media (DMEM containing 10% of the normal methionine concentration) for 3–4 h at 37°C. The cells were collected by centrifugation at 100 *g* for 5 min and washed two times in cold PBS. The cells were resuspended and solubilized for 15 min on ice in RIPA buffer (150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, and 50 mM Tris-HCl pH 8.0) containing 2 mM DTT and 1 μg/ml each of aprotonin, leupeptin, and pepstatin A. The extract was precleared of insoluble material by centrifugation at 100,000 *g* for 30 min at 4°C.

The RIPA soluble material from 10^7 ^{35}S-labeled HeLa cells was fractionated by chromatography on a Superose 12 HR10/30 gel filtration column (Pharmacia) equilibrated in RIPA buffer containing 0.5 mM DTT. 0.5-ml fractions were collected, and 1 μg/ml each of aprotonin, leupeptin, and pepstatin A were added to each fraction. These fractions were used for immunoadsorptions.

Immunoadsorptions

Anti-p97 mAb3E9 was prepared according to Chi et al. (1995). Mouse IgG was obtained from Sigma. Immobilized antibody-agarose was prepared by binding mAb3E9 or mouse IgG to goat anti–mouse IgG agarose beads in 50 mM Tris-HCl, pH 8.0, and 0.5 M NaCl at 1–2 mg antibody/ml beads. After two washes with the same buffer to remove unbound antibody, the beads were washed twice with 0.2 M sodium borate buffer, pH 9.0, and finally resuspended in 10 vol of the same buffer. Dry dimethyl pimelimidate was added to 20 μg/ml and incubated at room temperature for 30 min with mixing. The beads were then washed two times with 0.2 M glycine, pH 8.0, and stored in PBS containing 0.1% gelatin.

A RIPA buffer extract from 5×10^6 ^{35}S-labeled HeLa cells in a 1 ml vol was added to 20 μl of antibody beads and incubated at 4°C for 90 min. The beads were washed with RIPA buffer containing 0.5 mM DTT and 1 μg/ml each of aprotonin, leupeptin, and pepstatin A, and eluted with 20 μl of 1× sample loading buffer containing 40 mM DTT. Immunoadsorbed proteins were separated on 10% SDS-polyacrylamide gels and detected either by autoradiography or immunoblot. Specific proteins were detected after electrophoretic transfer to nitrocellulose membranes and blocking of nonspecific binding with 5% nonfat dry milk in TBST/M (50 mM Tris-HCl, 150 mM NaCl, 0.05% Tween-20, and 0.001% merthiolate) for 1 h at room temperature. After three 5-min washes with TBST/M, the following primary antibodies were used: affinity-purified rabbit anti-Ran/TC4 (1:2,000); rabbit anti-RanBP1 serum (1:1,000); rabbit anti-RanBP2 serum (1:1,000), affinity-purified rabbit anti-bovine NLS receptor polyclonal antibody (1:50), and rabbit anti-NPI serum (1:1,000) (O'Neill et al., 1995). Biotin-labeled anti–rabbit IgG (Vector Laboratories, Burlingame, CA) was used at 1 μg/ml as secondary antibody, and peroxidase-conjugated anti-biotin IgG was used at 1 μg/ml for detection. Detection on immunoblots was with luminol based chemiluminescence (Scheppenheim et al., 1991) and Kodak XAR5 film. For autoradiography, destained gels were treated with Enhance (New England Nuclear Research Products Boston, MA), dried under vacuum and exposed to Kodak XAR5 film at −80°C.

Solution Binding Assays

Partially purified S-tag p97 was adsorbed to 6 μl of S-protein agarose beads (Novagen) at 4°C for 1 h in binding buffer (20 mM Hepes, pH 7.3, 150 mM KOAc, 2 mM $Mg(OAc)_2$, 2 mM DTT, 0.1% Tween-20, 0.1% casamino acids, and 1 μg/ml each of aprotonin, leupeptin, and pepstatin A) as described (Rexach and Blobel, 1995). S-tag p97 beads were washed two times with binding buffer to remove unbound material and resuspended in 0.5 ml binding buffer. 2 μg of Ran-GDP or Ran-GTP and 2 μg of RanBP1 were added in various combinations as described in Results, and beads were incubated for 1 h at 4°C. For Ran-dissociation experiments, GST-p97 was bound to S-tag NLS receptor immobilized on S-protein agarose beads at 4°C for 1 h. Unbound material was removed with two washes of binding buffer and 2 μg Ran-GDP or Ran-GTP and RanBP1 were added as described in Results. The beads were then washed three times with binding buffer. Bound proteins were eluted with 50 μl 1× SDS-PAGE sample loading buffer and resolved by 12.5% SDS-PAGE (Dreyfuss et al., 1984). Proteins were detected by staining with Brilliant Blue R-250.

For the titration of Ran in solution binding assays, 2.5 μg of S-tag p97 immobilized on S-protein beads were incubated with 1.5 μg of RanBP1 and Ran as indicated in 0.5 ml binding buffer for 1 h at 4°C. Beads were washed three times with binding buffer to remove unbound material. Bound proteins, eluted from beads with 50 μl of 1× sample loading buffer, were resolved by 12.5% SDS-PAGE and visualized by staining with Brilliant Blue R-250. Quantitation of Ran was performed by laser scanning densitometry (LKB Instruments, Uppsala, Sweden).

Immunofluorescence Staining of Cells

HeLa JW36 cells were extracted and fixed as described (Chi et al., 1995). Rabbit anti-Ran antibody was diluted in 0.2% BSA in PBS at 25 μg/ml and incubated on the cells at room temperature for 1 h. After extensive washing in PBS, the antibody was detected with rhodamine-labeled goat anti–rabbit (Cappel Research Products, Durham, NC) diluted in the same buffer. The coverslips were mounted in 75% glycerol, 40 mM Tris-HCl, pH 8.0, and 0.1% *p*-phenylenediamine. The cells were observed by epifluorescence illumination as described above.

Results

Association of Nuclear Import Components with p97

Most aspects of nuclear protein import can be reconstituted in digitonin permeabilized cells with three purified or recombinant factors: the NLS receptor (importin α/karyopherin α), p97 (importin β/karyopherin β), and Ran/TC4 (Görlich and Mattaj, 1996). Immunoadsorptions of p97 from cell lysates with a specific monoclonal antibody (mAb3E9), identified other proteins that coimmunoadsorbed with p97 (Chi et al., 1995). To further characterize these proteins, immunoadsorptions with mAb3E9 were performed on cell extracts of ^{35}S-labeled HeLa cells prepared with various detergents. Extraction of cells with the detergent combination RIPA (see Materials and Methods) solubilized greater than 90% of the total cellular protein, including the glycosylated nucleoporins (data not shown). From this extract, three proteins with approximate molecular masses of 25, 30, and 350 kD were specifically coadsorbed with p97 (Fig. 1 *A*). These proteins had

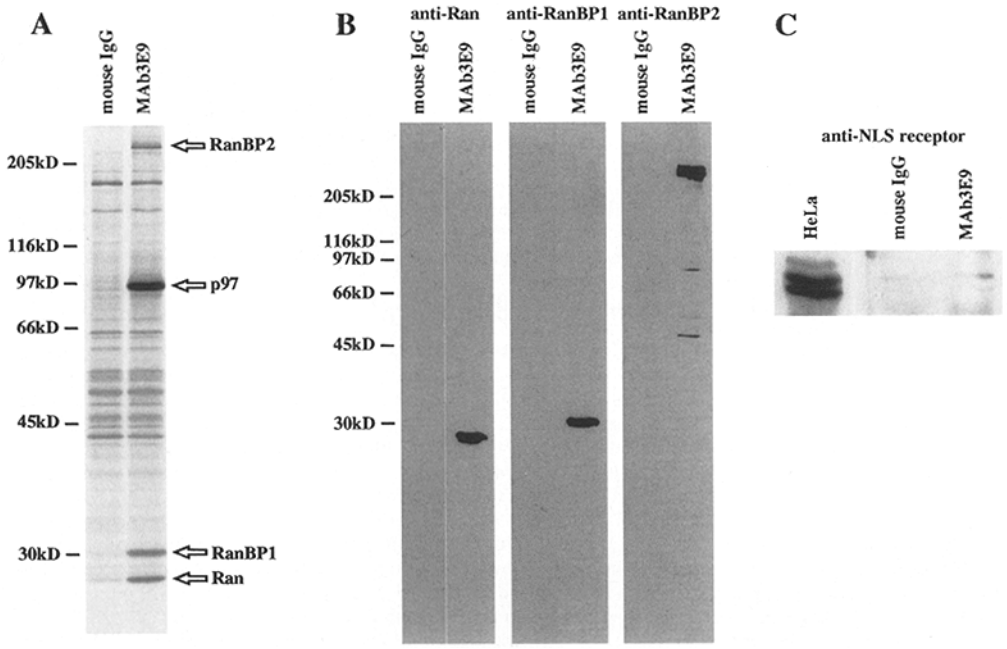

Figure 1. Immunoadsorptions and immunoblot analysis. (*A*) Immunoadsorptions of ^{35}S-labeled HeLa cell RIPA soluble extracts were performed as described in Materials and Methods with mAb3E9 or mouse IgG. Arrows indicate proteins specifically coadsorbed with p97. (*B*) Immunoadsorptions of HeLa cell RIPA soluble extracts and immunoblotting with antibodies to Ran/TC4, RanBP1, and RanBP2. (*C*) Immunoblot of total HeLa cell lysate, mAb3E9, and mouse IgG immunoadsorptions performed from HeLa cell RIPA soluble extracts with polyclonal antibodies to the NLS receptor homologue NPI.

similar electrophoretic mobilities to Ran/TC4, RanBP1, and RanBP2 (NUP358). Immunoblotting of the immunoadsorbed fractions with specific antibodies confirmed that Ran/TC4, RanBP1, and RanBP2 were specifically associated with p97 (Fig. 1 *B*). The immunoadsorbed proteins had identical electrophoretic mobilities when compared on autoradiograms and immunoblots. The 116-kD protein identified in our earlier study was not observed in these experiments because it was removed from mAb3E9 immunoadsorptions by washing with RIPA buffer (Chi, N., G. Visser, E. Adam, S. Adam, manuscript in preparation).

It has been suggested that p97 and the NLS receptor form a heterodimer that is responsible for targeting a karyophile to the nuclear pore (Adam and Adam, 1994; Enenkel et al., 1995; Görlich et al., 1995*a*; Imamoto et al., 1995; Moroianu et al., 1995*a*). No ^{35}S-labeled proteins with molecular weights corresponding to the NLS receptor were observed in the RIPA immunoadsorptions. Two homologues of the NLS receptor have been identified in human cells. Immunoblotting with antibodies to one of these homologues, NPI (O'Neill et al., 1995), did not detect NLS receptor among the specifically immunoadsorbed proteins, even though NPI was soluble in RIPA (Fig. 1 *C*). RIPA buffer was able to dissociate a NLS receptor-p97 complex assembled from the recombinant proteins in solution (data not shown), hence it was not surprising that the NLS receptor was absent from the immunoadsorptions.

To determine whether p97, Ran, and the Ran-binding proteins interact as a single complex or as several discrete complexes, RIPA soluble HeLa extracts were fractionated by gel filtration chromatography, and individual fractions were immunoadsorbed with mAb3E9 (Fig. 2). p97 was found in at least two distinct complexes. The first complex fractionated with an estimated molecular mass of >400 kD and contained p97, RanBP2, and Ran/TC4, although we cannot discount the possibility that the Ran in this complex is the leading edge of the Ran peak in the second complex. p97 has been shown to bind RanBP2, as well as other peptide repeat containing nucleoporins on overlay blots (Iovine et al., 1995; Moroianu et al., 1995*b*). Notably, none of the other peptide-repeat–containing nucleoporins were observed to bind p97 in these experiments, although they were soluble in the RIPA extract (data not shown). It is likely that the interaction of p97 with RanBP2 in RIPA buffer is stronger than its interaction with the other nucleoporins.

The second peak of p97 corresponded to an estimated molecular mass of 150–300 kD and contained p97, Ran, and RanBP1. By Coomassie blue staining, Ran and RanBP1 were present in approximately equimolar amounts. When the entire column elution profile was immunoblotted with anti-Ran antibodies, the majority of Ran was present in fractions eluting later in the column, representing molecular mass of 30–50 kD (data not shown). The amount of Ran present in the complex with p97 represents only a small percentage of the total Ran in the extract.

In Vitro Assembly of Ran, RanBP1, and p97

Recently, it was shown that Ran-GTP, but not Ran-GDP, binds directly to p97 (Rexach and Blobel, 1995; Floer and Blobel, 1996), and that Ran-GTP binding to p97 may require a Ran-binding domain from a Ran-binding protein for high affinity binding (Lounsbury et al., 1996). The simple interpretation of the immunoadsorption results in Fig. 1, then, was that a complex of p97, Ran-GTP, and RanBP1 was adsorbed from the cell lysate, with Ran-GTP linking p97 and RanBP1. To verify the formation of a Ran-RanBP1-p97 complex, we carried out direct binding experiments with the recombinant proteins. Bacterially expressed S-tag p97 immobilized on S-protein agarose beads was incubated with bacterially expressed Ran-GDP, Ran-GTP, and RanBP1. As expected, Ran-GTP bound p97 (Fig. 3 *A*), and the addition of RanBP1 in the incuba-

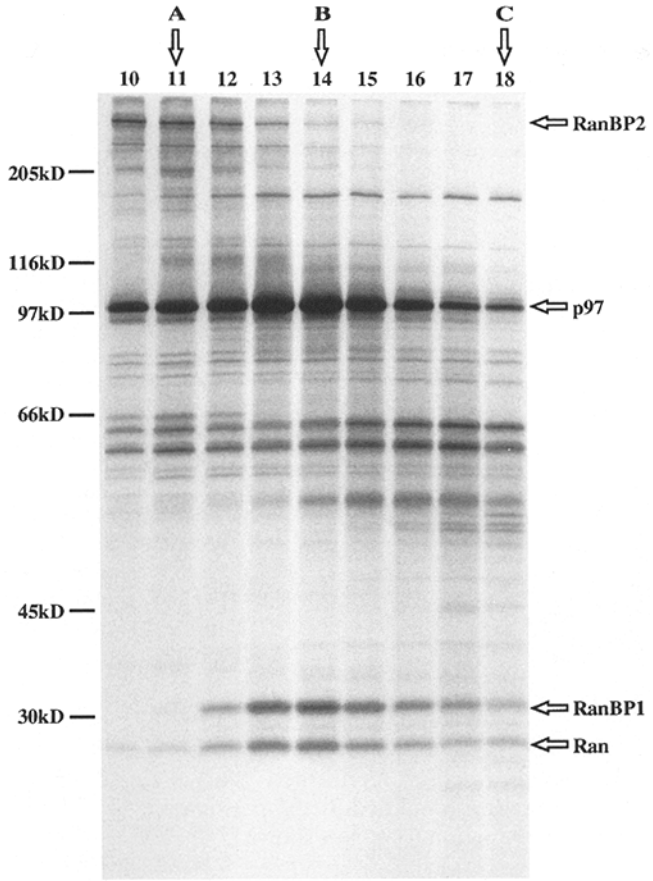

Figure 2. Gel filtration analysis of p97 complexes. ^{35}S-labeled HeLa cell RIPA soluble extract was fractionated by gel filtration chromatography. Individual gel filtration fractions were immunoadsorbed with mAb3E9. Size standards are indicated by vertical arrows above the fraction numbers. Size standards are (*A*) blue dextran, 400 kD; (*B*) β-amylase, 200 kD; and (*C*) IgG, 155 kD. Horizontal arrows indicate proteins that are coadsorbed specifically with mAb3E9. Molecular mass markers are indicated on the left.

tion with Ran-GTP and p97 led to the binding of RanBP1 and a slight increase in the amount of Ran-GTP bound. Neither Ran-GDP nor RanBP1 alone bound p97 under these conditions. However, when Ran-GDP and RanBP1 were added together, both Ran-GDP and RanBP1 associated with p97 to approximately the same degree as Ran-GTP and RanBP1. Ran charged with GMP-PNP binds p97 and RanBP1 in an identical manner as Ran-GTP (data not shown).

It has been suggested that one function of Ran-GTP in nuclear protein import is the dissociation of a p97-NLS receptor complex upon binding of Ran-GTP to p97 (Rexach and Blobel, 1995). To test if Ran-GDP/RanBP1 binding to p97 has the same complex-dissociating ability as Ran-GTP, we assembled a p97-receptor complex on immobilized NLS receptor (Fig. 3 *B*). As expected, the addition of Ran-GTP dissociated p97 from the immobilized receptor and this dissociation was not abrogated by the presence of RanBP1. Conversely, Ran-GDP and RanBP1 bound to the p97-receptor complex and did not dissociate p97 from the immobilized receptor.

The apparent increase in the affinity of Ran-GTP and Ran-GDP for p97 in the solution binding assays was verified by titration of Ran-GDP and Ran-GTP against fixed amounts of p97 and RanBP1 (Fig. 4). With RanBP1 present in a 1.5–2-fold excess over immobilized p97, Ran-GTP showed an approximately threefold increase in binding affinity for p97. An even more dramatic increase in affinity for p97 was seen for Ran-GDP. In the absence of RanBP1, Ran-GDP binding to p97 was undetectable even at a fourfold excess of Ran-GDP over p97 (Fig. 3 *A*). However, RanBP1 increased the affinity of Ran-GDP for p97 such that the affinities of Ran-GDP and Ran-GTP for p97 were indistinguishable.

RanBP1 Stabilizes the Receptor Complex on the Nuclear Pore

The nuclear pore binding, or docking, step in protein import can be reconstituted with purified or recombinant NLS receptor and p97 (Adam and Adam, 1994; Chi et al., 1995; Görlich et al., 1995*a*, Radu et al., 1995*a*). Pore binding showed a temperature dependence with a moderate decrease in binding when the temperature of incubation was increased from 4°C to 25°C (Fig. 5 *A*). The decrease in binding was accompanied by a slight accumulation of the APC-NLS within the nucleus, suggesting that residual Ran present in the permeabilized cells is capable of supporting transport at a basal level (Melchior et al., 1995*a*; Moore and Blobel, 1995). The inclusion of RanBP1 in the incubation at 4°C with p97 and receptor had little or no effect on binding of the APC-NLS to the pore. However, inclusion of RanBP1 at 25°C led to a dramatic increase in the amount of APC-NLS binding at the nuclear pore. At both 4°C and 25°C, RanBP1 stimulated small amounts of accumulation within the nucleus even in the absence of exogenous Ran (Fig. 5 *A*). The extent of intranuclear accumulation with RanBP1 was higher at 25°C than at 4°C in ~30% of the cells. This RanBP1 stimulated accumulation suggests that the Ran present on the pore in permeabilized cells must be available for limited rounds of transport. RanBP1 was unable to substitute for either the NLS receptor or p97 under any of the above conditions (Fig. 5 *B*), indicating that it was acting as a distinct factor in the assay.

Saturation of nuclear pore binding in the permeabilized cell assay was reached at 300 nM NLS receptor and 150 nM p97 when assayed at 4°C on 2×10^6 HeLa cells (Fig. 6). The extent of binding was dependent also on the number of cells present in the assay; that is, when a greater number of cells was used for binding, an increased concentration of the import factors was required to achieve saturation (data not shown). When the incubation was carried out at 25°C, nuclear pore binding was decreased by ~40% relative to incubations at 4°C at all concentrations of the two binding factors (Fig. 6). Inclusion of RanBP1 at 500 nM in the binding assays increased karyophile binding to the pore at 25°C to 80–90% of the level observed at 4°C, but had no significant effect at 4°C. The temperature dependence of docking suggested that RanBP1 stabilized the interaction of the receptor complex with the pore and exerted its effects on an energy using transport factor, presumably Ran, that is present at the pore complex.

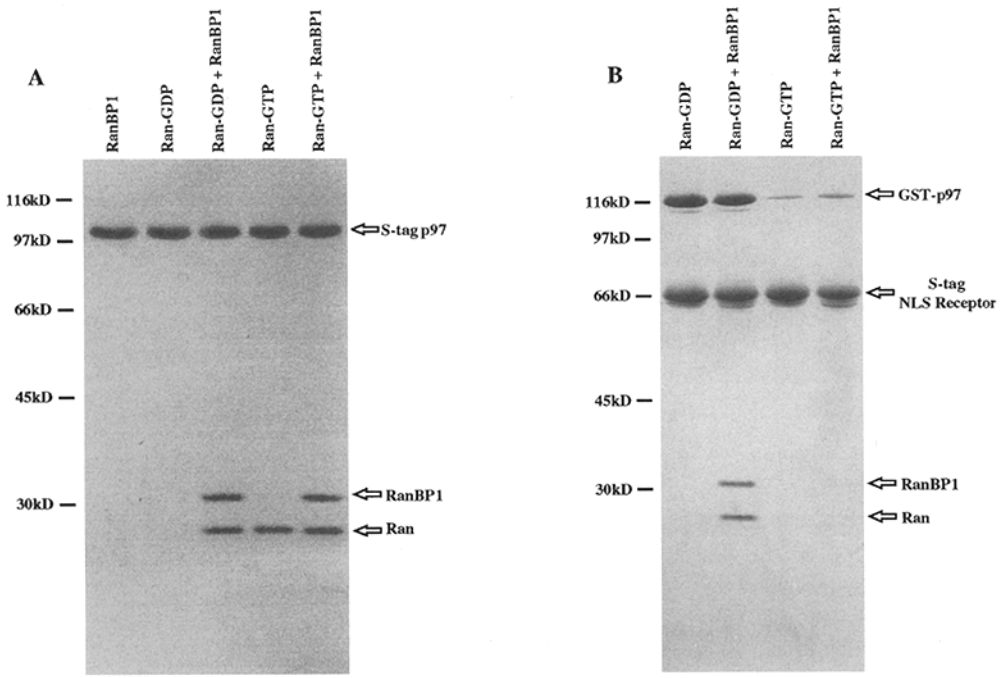

Figure 3. Assembly of complexes with recombinant transport factors. (*A*) S-protein agarose beads bound with S-tag p97 were incubated with recombinant Ran-GDP, Ran-GTP, and RanBP1 at various combinations as indicated. (*B*) S-protein agarose beads bound with S-tag NLS receptor were incubated with Ran-GDP, Ran-GTP, RanBP1, and GST-p97 at various combinations as indicated.

Localization of Ran on the Nuclear Envelope in Permeabilized Cells

Previous studies have suggested that digitonin permeabilization of cultured cells releases >90% of Ran from the cell (Görlich et al., 1995*b*; Melchior et al., 1995*a*; Moore and Blobel, 1995). To verify that our permeabilized cells contained endogenous Ran, we performed indirect immunofluorescence with anti-Ran antibodies on HeLa cells. As expected, a small but detectable concentration of Ran was found at the nuclear envelope (Fig. 7) (Moore and Blobel, 1995). Immunoblotting of digitonin soluble and insoluble cell fractions confirmed that a small amount (<10%) of Ran remained in the permeabilized cell after digitonin extraction (data not shown).

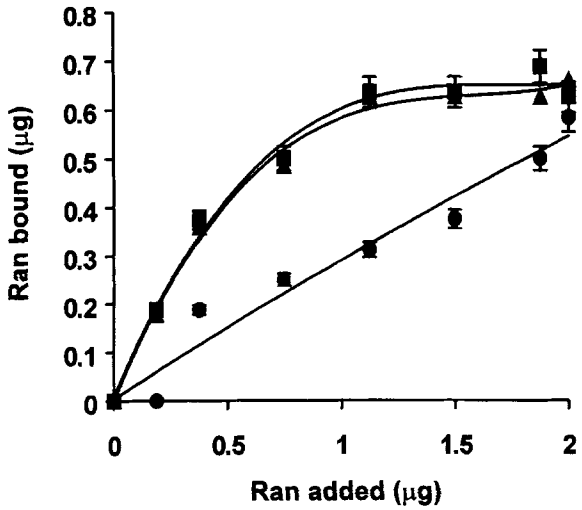

Figure 4. Affinity of Ran-GTP and Ran-GDP for p97. Solution binding assays were carried out as in Fig. 3 and the amount of Ran-GTP or Ran-GDP bound was determined. The data set for Ran-GDP binding is not shown because no binding could be detected. (●, Ran-GTP; ▲ Ran-GDP + RanBP1; ■, Ran-GTP + RanBP1.)

RanBP1 Stimulates Nuclear Protein Import

The import of proteins into the nucleus can be reconstituted in permeabilized cells with the NLS receptor, p97, and Ran in the presence of ATP and GTP (Melchior et al., 1993; Moore and Blobel, 1993). A fourth factor, p10/NTF2, has been suggested to stimulate nuclear protein import under the conditions used here (Moore and Blobel, 1994; Paschal and Gerace, 1995), but recombinant human NTF2 had no effect in our assays, and was not included (data not shown). Because of the stabilizing effect of RanBP1 on the association of a karyophilic protein with the pore complex and the stimulation of small amounts of transport in the docking assay (Fig. 5 *A*), RanBP1 was added along with NLS receptor, p97, and Ran-GDP in the presence of ATP and GTP to determine its effect on translocation into the nucleus. The addition of RanBP1 to 1 μM in the transport reaction increased the level of intranuclear APC-NLS accumulation by 40–50% over reactions without RanBP1 (Fig. 8 *A*). Titration of Ran and RanBP1 demonstrated that the extent of APC-NLS accumulation was stimulated by RanBP1 at all concentrations of Ran tested (Fig. 8, *A* and *B*). As shown also in Fig. 5, RanBP1 stimulates import in the absence of exogenous Ran (Fig. 8 *B*). As expected from previous results, the final extent of import was dependent upon the amount of Ran present in the assay (Melchior et al., 1995*a*).

Discussion

A number of proteins have been identified as components of the nuclear protein import machinery. These proteins

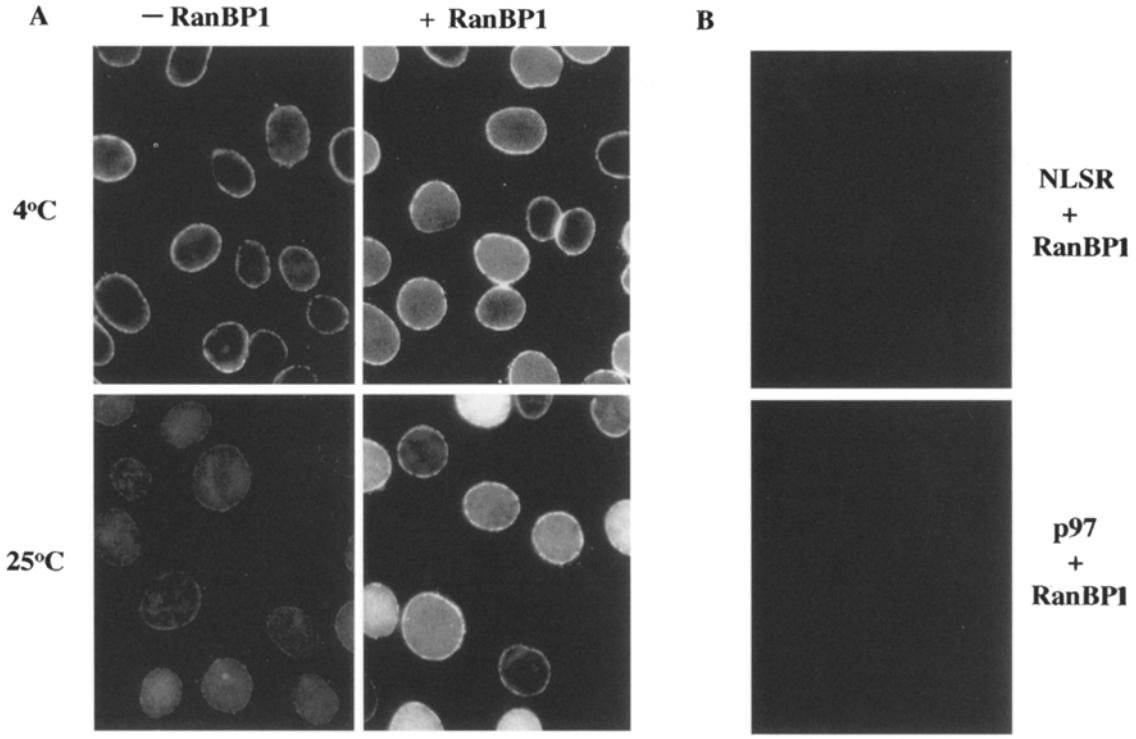

Figure 5. RanBP1 stabilizes nuclear pore binding. (*A*) Nuclear pore binding assays were performed at 4°C or 25°C with 100 nM p97 and 200 nM NLS receptor (NLSR) or 100 nM p97, 200 nM NLS receptor, and 500 nM RanBP1. (*B*) Nuclear pore binding assays were performed at 4°C with 200 nM NLS receptor and 500 nM RanBP1 or 100 nM p97, and 500 nM RanBP1.

include the 54/56-kD NLS receptor/importin α/karyopherin α_1 and α_2, p97/importin β/karyopherin β, Ran/TC4, NTF2/p10, and hsc70/hsp70 (Powers and Forbes, 1994). Although import can be reconstituted in permeabilized cells with the NLS receptor, p97 and Ran/TC4, the relatively high concentrations needed for import in vitro suggests that other factors may be involved. Some likely candidates for these unknown factors have been identified by their interaction with p97 (Chi et al., 1995; Imamoto et al., 1995) or Ran/TC4 (Lounsbury et al., 1994; Saitoh and Dasso, 1995). Here we demonstrate a biochemical and functional interaction between p97, Ran/TC4, and RanBP1 and a biochemical interaction between p97, Ran/TC4, and RanBP2.

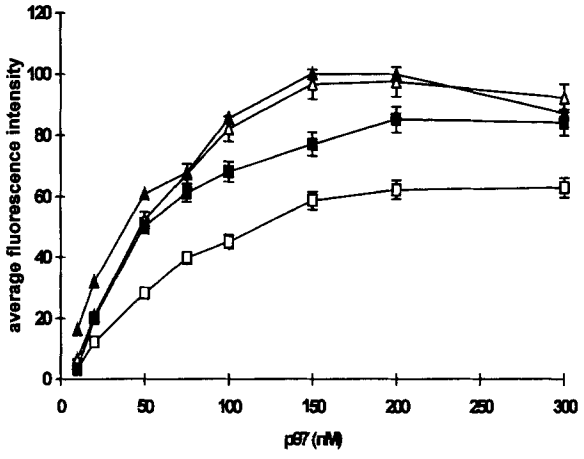

Figure 6. Quantitative analysis of RanBP1 stabilizing activity. Increasing concentrations of NLS receptor and p97 were included in the pore binding assay as described in Materials and Methods at the following conditions: △, 4°C; ▲, 4°C and 500 nM RanBP1; □, 25°C; ■, 25°C and 500 nM RanBP1. Average fluorescence intensity of the nuclear envelope was measured as described in Materials and Methods.

p97 Forms Specific Complexes with Ran, RanBP2, and RanBP1

p97 was originally identified as one of two proteins required to target NLS-containing proteins to the nuclear pore (Adam and Adam, 1994). A heterodimer composed of p97 and the NLS receptor is believed to form a cytoplasmic receptor complex that docks karyophiles onto the nuclear pore as an early step in import (Adam and Adam, 1994; Görlich et al., 1995*a*; Enenkel et al., 1995; Imamoto et al., 1995). Since p97 alone can bind to the nuclear pore complex, specifically the peptide repeat region of nucleoporins (Görlich et al., 1995*b*; Iovine et al., 1995; Moroianu et al., 1995), it is likely that the docking event is mediated by p97 through binding to a nucleoporin. Nup358, a peptide-repeat containing nucleoporin, also known as RanBP2, localizes to the cytoplasmic filaments of the nuclear pore complex and forms strong interactions with p97 and Ran/TC4 (Melchior et al., 1995*a*; Moroianu et al., 1995*b*; Wu et al., 1995; Yokoyama et al., 1995). RanBP2 has been suggested to be the site of Ran-GTP binding and GTP hydrolysis at the pore during protein import. Binding of the receptor complex and Ran-GTP to RanBP2 may be the initial and critical point for regulating nuclear protein import (Melchior et al., 1995*a*). In our experiments,

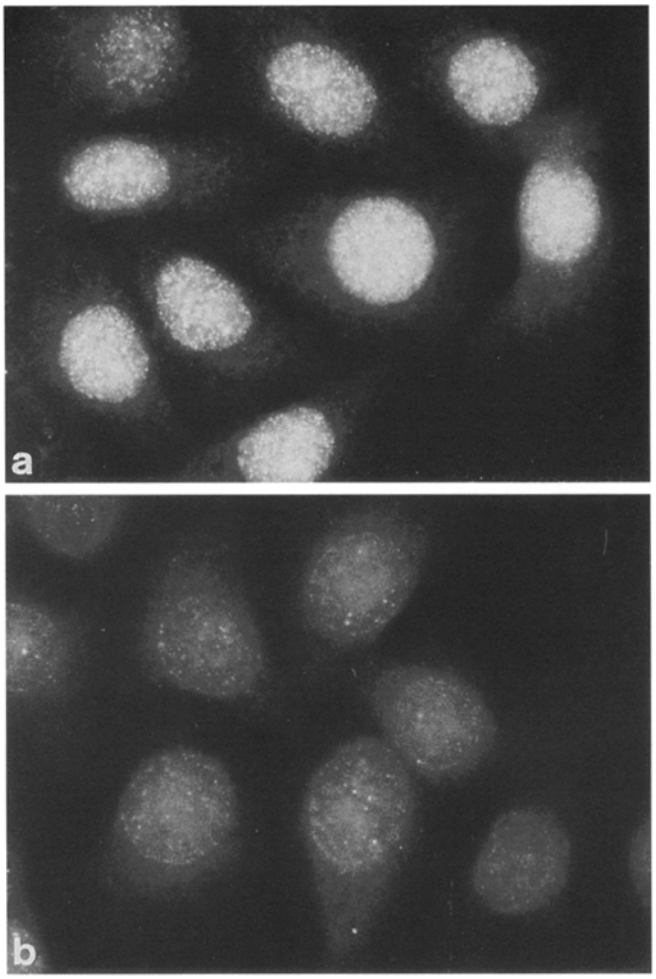

Figure 7. Localization of Ran in permeabilized cells. Ran was localized in HeLa cells with anti-Ran antibodies. (*a*) Cells were fixed with formaldehyde before extraction with Triton X-100. (*b*) Cells were permeabilized with digitonin before fixation and extraction as in *a*.

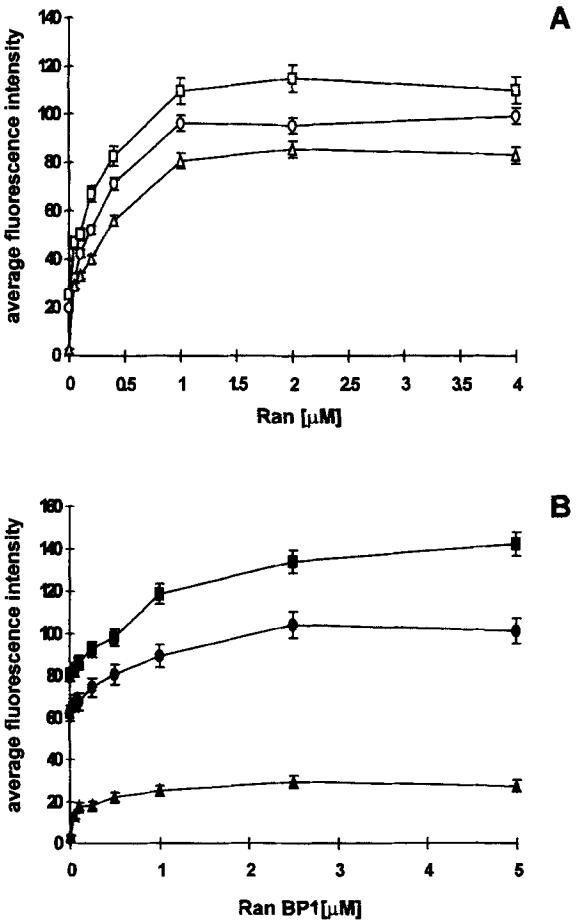

Figure 8. Quantitative analysis of nuclear protein import stimulation by RanBP1. (*A*) Ran was titrated against fixed amounts of NLS receptor and p97 at various levels of RanBP1 as indicated. (*B*) RanBP1 was titrated against fixed amounts of NLS receptor and p97 at three concentrations of Ran as indicated. (▲, 0 mM Ran; ●, 1 μM Ran; ■, 2 μM Ran; △, 0 μM RanBP1; ○, 1 μM RanBP1; □, 2 μM RanBP1.)

RanBP2 is the only peptide repeat containing nucleoporin that forms a stable complex with p97 in RIPA buffer. The RIPA soluble complex of p97-RanBP2-Ran may represent a portion of the initial docking complex extracted from the pore, with the NLS receptor having been removed by the detergent. The high affinity of the p97-RanBP2 binding may reflect the importance of this interaction as the first point of association of the receptor complex with the pore. As the first site of interaction with the pore, RanBP2 could serve to facilitate the assembly of transport complexes by concentrating Ran and receptor complexes at the cytoplasmic side of the pore. It is interesting to note that p97 associates with RanBP1 and RanBP2 independently. Substituting Ran-GDP-RanBP1 for RanBP2 on p97 after the initial docking event may disrupt the association of the receptor complex with RanBP2.

Ran/TC4 Binding to p97

Conflicting results on the binding of Ran-GTP to p97 have been reported recently. In solution binding assays, Ran-GTP exhibited high affinity binding to p97, but Ran-GDP did not bind (Rexach and Blobel, 1995; Floer and Blobel, 1996). However, on overlay blots of p97 in cell lysates, Ran-GTP had a low affinity for p97 that was enhanced by the Ran-binding domain of RanBP1 or RanBP2 (Lounsbury et al., 1996). In our hands, Ran-GTP binds p97 with high affinity, and the affinity is enhanced by the binding of RanBP1. In addition, RanBP1 and Ran-GDP bind p97 cooperatively since the binding for either protein to each other or to p97 cannot be detected in the solution binding assay. The affinity of p97 for Ran-GTP or Ran-GDP when bound to RanBP1 is similar and is higher than the affinity of p97 for Ran-GTP alone.

A conserved acidic COOH-terminal domain (-DED-DDL) of Ran is required for the high affinity binding of Ran-GTP to RanBP1 (Lounsbury et al., 1994; Ren et al., 1995; Richards et al., 1995). The acidic sequence in Ran affects the role of RanBP1 as a costimulator of RanGAP (Richards et al., 1995). It has been suggested that GTP binding to Ran causes a conformational change in the protein exposing the acidic domain for protein–protein interactions. In Ran-GDP, this domain may be folded into the

guanine nucleotide binding pocket, stabilizing GDP binding and preventing the acidic domain from interacting with RanBP1 (Richards et al., 1995). p97 contains a conserved acidic sequence (^{334}DENDDDDDWN^{343}) similar to that found in Ran. This acidic domain is located in the portion of p97 that binds Ran (Chi, N., and S. Adam, manuscript in preparation) and may interact with Ran-GDP exposing the acidic domain of Ran to RanBP1 leading to the cooperative formation of a trimeric Ran-GDP-RanBP1-p97 complex.

RanBP1 Stabilizes the Receptor Complex on the Nuclear Pore

The results presented here suggest a role for Ran in the association of the receptor complex with the nuclear pore. This conclusion is supported by the observation that nonhydrolyzable analogues of GTP inhibit docking as well as translocation in permeabilized cells (Melchior et al., 1993; Moore and Blobel, 1993). The stabilization of docking by RanBP1 in our assays and the localization of Ran to the nuclear envelope suggests that Ran is associated with the docking site in permeabilized cells. At least two nucleoporins, RanBP2 and Nup2p, contain Ran-binding domains (Dingwall et al., 1995) and several additional Ran-binding proteins have been identified in the high salt/detergent fraction of nuclei (Lounsbury et al., 1994). RanBP1 may promote docking by stabilizing the binding of the receptor complex at the docking site when Ran is in the GDP form. An alternative possibility is that the p97-NLS receptor-Ran-GDP-RanBP1 complex characterized here is the actual translocation complex that moves through the pore. This assembly may form after the release of the receptor complex and Ran-GDP from RanBP2 upon GTP hydrolysis. A RanBP1 stabilized receptor complex would be able to undergo repeated binding and dissociation events from downstream docking sites without disassembling. At some point after docking, an exchange reaction to replace the GDP with GTP on Ran must occur, catalyzed by a yet to be identified factor. Ran-GTP would release the receptor complex from the docking site, but to prevent disassembly of the receptor complex, the Ran-GTP would have to be hydrolyzed rapidly. RanBP1 functions as a coactivator of RanGAP1 and would be useful in increasing the rate of GTP hydrolysis upon release of the receptor complex from the docking site (Bischoff et al., 1995).

p10/NTF2 has been proposed to associate Ran-GDP with docked karyopherin αβ (receptor complex) to form a short-lived pentameric docking complex containing a nucleoporin, p97, NLS receptor, p10, and Ran-GDP (Nehrbass and Blobel, 1996). From that study, it is not clear if p10 aids in the association of Ran-GDP with p97 (karyopherin β), the nucleoporin or both, since p10 binds to both proteins and to Ran-GDP. RanBP1 has a similar effect to p10 in the solution binding assay as it promotes the binding of Ran-GDP to p97 without dissociating the p97-receptor heterodimer. In contrast to p10, RanBP1 binds Ran-GTP with high affinity (Coutavas et al., 1993; Lounsbury et al., 1994). The addition of GTP to the p10-docked complex led to a gradual partial dissociation of the karyopherin heterodimer, an effect not seen with RanBP1 complexes in solution binding assays (data not shown) or docking assays in permeabilized cells. In our hands, recombinant mammalian p10/NTF2 has no effect on transport or docking in permeabilized cells and does not bind p97 with high affinity in the solution binding assay (Chi, N., and S. Adam, unpublished results).

On the Mechanism of Translocation Through the Pore

A model to explain movement of karyophiles through the nuclear pore has been proposed, based on the results of solution binding experiments with recombinant transport factors (Rexach and Blobel, 1995). This model suggests that a series of docking, undocking, diffusion, and redocking events occur in a stochastic process leading to translocation through the pore. In this model, association of the receptor complex with the peptide repeats of the nucleoporins dissociates the karyophile from the receptor-p97 heterodimer, while binding of Ran-GTP to p97 in the complex dissociates p97 from the receptor and the nucleoporin. Thus, at each docking event, the model proposes that the entire receptor complex disassembles, only to reassemble immediately at another docking site. This model is inconsistent with the observations that stable docking occurs in permeabilized cells (Adam and Adam, 1994; Görlich et al., 1995*a*; Enenkel et al., 1995; Imamoto et al., 1995) and the transport factor-mediated binding of karyophiles to nucleoporins on overlay blots does not disrupt the receptor complex (Radu et al., 1995*a*; Iovine et al., 1996; Moroianu et al., 1995*b*). Such a dissociation reaction would be useful at the final step in transport when release of the karyophile into the nucleoplasm and recycling of the transport factors are required.

Stabilization of a receptor complex by RanBP1 upon docking explains some of the apparent discrepancies between this model and results in the literature. A stabilizing role for RanBP1 is also consistent with the observed increase in transport efficiency with RanBP1 in permeabilized cells. Without stabilization of the complex during each docking and release cycle, complete disassembly of the receptor complex (Rexach and Blobel, 1996) would decrease the efficiency of the transport process. By allowing Ran to remain associated with the receptor complex, RanBP1 would position Ran for efficient utilization by the transport machinery without a dependence on multiple dissociation-association events. A terminal binding event, similar to the binding of the receptor complex to RanBP2, may remove RanBP1 from the complex by competition for the same binding domain. This would be a useful mechanism for the final dissociation of the receptor complex and release of the karyophile at the nucleoplasmic basket of the pore (Rexach and Blobel, 1995).

It should be considered that some components of the import pathway may not be required in permeabilized cells when an excess of another factor is present (Görlich et al., 1994). This explains the stimulatory effects of nonrequired factors such as p10/NT2 and RanBP1 in permeabilized cells. These accessory factors lower the requirements for the three required transport factors and increase their efficiency at lower concentrations. In addition, docking in permeabilized cells may occur at multiple sites along the suggested array of docking sites in the pore. A primary

docking site may be by-passed if the concentration of transport factors is sufficiently high to overcome the lower affinity interactions of the factors with secondary docking sites. Such complications of the permeabilized cell assay must be considered when model building, until more of the biochemical interactions between the transport factors are understood. It will be important to characterize the activities of the transport factor-associated proteins that have been identified to understand the process of nuclear protein import in greater detail.

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