

# GTP Hydrolysis by Ran Occurs at the Nuclear Pore Complex in an Early Step of Protein Import

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**Abstract.** Mediated import of proteins into the nucleus involves multiple cytosolic factors, including the small GTPase Ran. Whether Ran functions by interacting with other cytosolic proteins or components of the nuclear pore complex has been unclear. Furthermore, the precise transport step where Ran acts has not been determined. To address these questions, we have analyzed the binding interactions of Ran using permeabilized cells and isolated nuclear envelopes. By light and electron microscope immunolocalization, we have found that Ran accumulates specifically at the cytoplasmic surface of the nuclear pore complex when nuclear import in permeabilized cells is inhibited by nonhydrolyzable analogs of GTP. Ran associates with a peripheral pore complex region that is similar to the area where transport ligands accumulate by depletion of

ATP, which arrests an early step of transport. Binding studies with isolated nuclear envelopes in the absence of added cytosol indicate that Ran-GTP directly interacts with a pore complex protein. Using blot overlay techniques, we detected a single prominent polypeptide of isolated nuclear envelopes that binds Ran-GTP. This corresponds to the 358-kD protein RanBP2, a Ran binding pore complex protein recently identified by two-hybrid screening. Thus, RanBP2 is likely to constitute the Ran-GTP-binding site detected at the cytoplasmic periphery of the pore complex. These data support a model in which initial ligand binding to the nuclear pore complex occurs at or near RanBP2, and that hydrolysis of GTP by Ran at this site serves to define commitment to the nuclear import pathway.

**R**<sub>AN</sub> defines an evolutionarily highly conserved branch of the Ras superfamily of low molecular weight GTPases that is found in both the nucleus and cytoplasm (Drivas et al., 1990; Bischoff and Ponstingl, 1991a). Unlike other Ras-like GTPases, Ran is not post-translationally modified by lipid. The guanine nucleotide state of Ran is modulated by a number of interacting proteins, including the guanine nucleotide exchange factor RCC1 (Ohtsubo et al., 1987; Bischoff and Ponstingl, 1991b) and the GTPase activating protein RanGAP1 (Bischoff et al., 1994). Two additional Ran-GTP binding proteins have been identified recently, the 24 kD protein RanBP1 (Coutavas et al., 1993; Bischoff et al., 1995b) and the 358-kD protein RanBP2/Nup358 (Yokoyama et al., 1995; Wu et al., 1995). The phenotypes associated with defects in Ran and its interacting proteins are highly pleiotropic and relate to diverse cellular functions that include cell cycle checkpoint control, RNA processing and export, maintenance of nuclear structure and nuclear protein import (reviewed in Dasso, 1993; recent studies include, e.g., Kornbluth et al., 1994; Ouspenski et al., 1995; Cheng et al.,

1995). With one exception (below) it is unclear whether these functions are influenced directly or indirectly by Ran. A direct connection between Ran and nuclear protein import has been established recently with the use of in vitro assays, where it has been shown that nuclear import is dependent on the presence of Ran and that Ran is responsible for inhibition of nuclear import by nonhydrolyzable GTP analogs (Melchior et al., 1993a; Moore and Blobel, 1993). Supporting these results, recent studies found that the expression of a permanently activated Ran mutant in *Saccharomyces cerevisiae* leads to import defects (Schlenstedt et al., 1995) and that the temperature sensitive hamster cell line tsBN2 defective for RCC1 (Ohtsubo et al., 1987) develops protein import defects at the restrictive temperature (Tachibana et al., 1994).

Transport across the nuclear envelope (NE)<sup>1</sup> is mediated by elaborate supramolecular structures called nuclear pore complexes (NPCs) (reviewed by Forbes, 1992; Fabre and Hurt, 1994; Panté and Aebi, 1994; Rout and Wentz, 1994; Melchior and Gerace, 1995). Ions and small metabolites can diffuse passively through 10-nm diameter aque-

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1. *Abbreviations used in this paper:* MBS, *m*-maleimido-benzoyl-*N*-hydroxy-succinimide ester; NE, nuclear envelope; NLS, nuclear localization sequence; NPC, nuclear pore complex; RT, room temperature.

ous channels that span the NPC, but most proteins and RNAs are too large to diffuse efficiently through these channels. Instead, most macromolecules are transported through a gated channel in the center of the NPC by signal, ATP and temperature-dependent mechanisms. Import of karyophilic proteins is specified by nuclear localization sequences (NLSs), which usually have a highly basic character, on the transported proteins and appears to involve multiple sequential steps. These are proposed to include binding of the transport ligand containing an NLS to a cytoplasmic NLS receptor, association of this ligand/receptor complex with the periphery of the NPC, movement of the ligand/receptor complex to the central gated channel, translocation through the gated channel into the nuclear interior, and recycling of the receptor to the cytoplasm (discussed in Melchior and Gerace, 1995). Analysis of NLS-mediated nuclear import has been greatly promoted by the development of assays that reconstitute this process *in vitro*. We have developed a system consisting of digitonin permeabilized mammalian cells supplemented with a fluorescent transport ligand, an ATP regenerating system, and exogenous cytosol (Adam et al., 1990; Paschal and Gerace, 1995). Biochemical manipulation of permeabilized cell systems has enabled identification of five different cytosolic factors required for nuclear import: the NLS receptor/importin 60/karyopherin  $\alpha$ /hSRP1 $\alpha$  (Adam and Gerace, 1991; Görlich et al., 1994; Moroianu et al., 1995; Weis et al., 1995), p97/importin 90/karyopherin  $\beta$  (Adam and Adam, 1994; Chi et al., 1995; Görlich et al., 1995; Radu et al., 1995), Hsp70 (Imamoto et al., 1992; Shi et al., 1992), NTF2/p10 (Paschal and Gerace, 1995; Moore and Blobel, 1994a), and the Ras-related GTPase Ran (Moore and Blobel, 1993; Melchior et al., 1993a).

The transport steps mediated by some of these factors are beginning to be characterized in detail. The NLS receptor together with p97 appear to be sufficient to mediate fluorescent ligand accumulation at the NE in permeabilized cells, as visualized by light microscopy (Adam and Adam, 1994; Moroianu et al., 1995; Görlich et al., 1995). A similar pattern of ligand accumulation at the nuclear rim is seen both *in vivo* and *in vitro* when transport is inhibited by ATP depletion (Richardson et al., 1988; Newmeyer and Forbes, 1988). At present, it is unknown whether binding of ligand to the NE under these conditions involves the ~30–50-nm-long fibrils emanating from the cytoplasmic surface of the NPC (Jarnik and Aebi, 1991), which are thought to be the initial sites of ligand binding at the NPC *in vivo* (Richardson et al., 1988). Studies with Ran and NTF2/p10 (Moore and Blobel, 1994a; Paschal and Gerace, 1995) have suggested that these factors function at a stage subsequent to initial binding of ligand at the NE.

Since GTPases generally act as molecular switches (Bourne et al., 1990), an issue of major interest has been the nature of the transport step regulated by hydrolysis of GTP by Ran. Since exogenously added Ran is not required for the accumulation of NLS ligands at the NE (e.g., Moore and Blobel, 1993; Adam and Adam, 1994; Görlich et al., 1995) and ligand prebound to the NE in the absence of Ran can be dissociated from it by the addition of Ran and p10/NTF2 (Moore and Blobel, 1994a), it has been suggested that Ran is involved in the transport step involving translocation into the nucleus, and that hydroly-

sis of GTP by Ran occurs within the nucleus to release transport ligand from its carrier (Moore and Blobel, 1994b). In contrast, the findings presented here indicate that GTP hydrolysis by Ran is part of an early transport step that occurs near the site of initial ligand binding. We propose that hydrolysis of GTP by Ran serves to commit transport ligand to the nuclear import pathway. The implications of this model related to the vectoriality and rate of nuclear protein import are discussed.

## Materials and Methods

### Cells, Reagents, and Antibodies

Suspension cultures of human (HeLa) cells were grown in Joklik's-modified minimal essential medium (GIBCO BRL, Gaithersburg, MD) with 10% newborn calf serum (Hyclone Laboratories, Logan, UT). Nucleotide stock solutions (with equimolar Mg-acetate in 50 mM Hepes, pH 7.4) were stored in liquid nitrogen. Digitonin (high purity), creatine phosphate and creatine phosphokinase were obtained from Calbiochem-Behring Corp. (San Diego, CA). A 10% stock solution of digitonin in DMSO was stored in aliquots at  $-20^{\circ}\text{C}$ .

Based on a protocol described by Ren et al. (1993), polyclonal rabbit  $\alpha$ -Ran antibodies were raised against a synthetic peptide comprising the hypervariable COOH-terminal region of Ran: QYEHDLVAQTT (amino acids 196–207). The synthetic peptide was coupled via an additional cysteine at the NH<sub>2</sub> terminus of the peptide to the carrier protein ovalbumin using the bifunctional crosslinker *m*-maleimido-benzoyl-*N*-hydroxy-succinimide ester (MBS). Rabbits were immunized using the peptide-ovalbumin conjugate emulsified with Titermax<sup>R</sup> (Vaxcel<sup>TM</sup>, Inc., Nerecess, GA) for the initial subcutaneous injection and emulsified with incomplete Freund's adjuvant for subsequent booster injections (8, 13, 18 wk after the initial immunization). Rabbits were routinely bled 10 d after the booster injections. Specific antibodies were adsorbed to an affinity chromatography column containing the Ran peptide coupled via MBS to EAH Sepharose 4B (Pharmacia, Piscataway, NJ), eluted with a solution containing 0.2 M acetic acid, pH 2.7, and 0.5 M NaCl, dialyzed against PBS, concentrated, and stored at  $-20^{\circ}\text{C}$ . Affinity purified  $\alpha$ -RanBP2 peptide antibodies (551) that were raised against a sequence (SKAPKSG-FEGMFTKK) repeated three times in the zinc finger motifs of RanBP2 are characterized by Yokoyama et al. (1995). RL1 monoclonal antibodies against a group of O-linked glycoproteins of the NPC were characterized previously (Snow et al., 1987; Holt et al., 1987). Rabbit  $\alpha$ -BSA antibody preparations were obtained from Calbiochem-Behring Corp. (San Diego, CA) and purified by chromatography on Protein G Sepharose. Secondary antibodies for immunoblots (peroxidase-conjugated goat  $\alpha$ -rabbit IgG, or alkaline phosphatase-conjugated donkey  $\alpha$ -rabbit IgG) were obtained from Pierce (Rockford, IL), secondary antibodies for immunofluorescence microscopy (rhodamine-conjugated donkey  $\alpha$ -rabbit IgG) were from Jackson ImmunoResearch Laboratories (West Grove, PA), and 5 nm colloidal gold-conjugated secondary antibodies (goat  $\alpha$ -rabbit IgG) were obtained from Zymed (San Francisco, CA).

### In Vitro Import Assay

Digitonin permeabilized cells and cytosol were prepared as described (Melchior et al., 1995). Import assays and preparation of transport ligand were carried out as described in Paschal and Gerace (1995). All assays contained 2.5 mg/ml cytosol, an ATP regenerating system (1 mM ATP, 5 mM creatine phosphate, 20 U/ml creatine phosphokinase), transport ligand (0.25  $\mu\text{M}$  FITC-BSA conjugated with synthetic peptides containing the SV-40 wild type nuclear localization sequence) and  $3 \times 10^5$  permeabilized cells. For inhibition of transport, these mixes were supplemented with either 200  $\mu\text{M}$  GMP-PNP, or 16 U Hexokinase/5 mM glucose. Transport reactions, carried out in 6 ml polystyrene tubes, were started by transfer into a  $30^{\circ}\text{C}$  water bath. Transport was arrested after 30-min incubation by dilution with 4 ml 3.7% formaldehyde in transport buffer. After fixation at room temperature for 10 min, cells were washed once with transport buffer, and processed further for immunofluorescence or immunogold staining (below).

## Detection of Ran and Ligand after In Vitro Import Assays

**Indirect Immunofluorescence Microscopy.** Fixed cells derived from in vitro import assays were harvested by centrifugation and mounted on poly-L-lysine-coated coverslips. After permeabilization with 0.2% Triton X-100 for 5 min the cells were incubated with 5  $\mu\text{g/ml}$   $\alpha$ -Ran antibodies in PBS and 2% BSA for 2 h at room temperature (RT), washed twice with PBS, incubated for 1 h at RT with rhodamine conjugated donkey  $\alpha$ -rabbit antibodies (diluted 1:300), washed again, and mounted with SlowFade<sup>TM</sup> (Molecular Probes, Inc., Eugene, OR) onto microscope slides. For each sample, FITC-(ligand)- and rhodamine-(Ran)-derived signals were photographed with a Zeiss Axiophot microscope and Kodak TMAX 400 film. Within each experiment, exposure time, film processing, and printing were identical for all images.

**Electron Microscopy.** To localize Ran and the transport ligand in permeabilized cells after various transport conditions, fixed cells were harvested by centrifugation, and incubated (without Triton X-100 treatment) for 2 h at RT with the respective primary antibody (5  $\mu\text{g/ml}$   $\alpha$ -Ran antibodies or 17  $\mu\text{g/ml}$   $\alpha$ -BSA IgGs) in PBS and 2% ovalbumin, washed, and subsequently incubated for 3 h at RT or overnight at 4°C in PBS and 2% Ovalbumin with  $\alpha$ -rabbit-IgG antibodies conjugated to 5 nm colloidal gold particles (dilution 1:25). The cells were then fixed either with 2% glutaraldehyde in PBS containing 0.02% tannic acid or with 2% glutaraldehyde in 0.1 M sodium cacodylate, pH 7.2, 0.02% tannic acid, post-fixed with 1% osmium tetroxide, dehydrated, and embedded for electron microscopy. Thin sections were stained with 2% uranyl acetate for 1 min.

## Expression, Purification, and Nucleotide Loading of Ran/TC4

Ran, expressed in *Escherichia coli* BL21(DE3), was purified as described (Melchior et al., 1995). To load recombinant Ran with nonradioactive nucleotides (GTP, GDP, or GMP-PNP), 20  $\mu\text{M}$  Ran was incubated for 30 min at 20°C with 1 mM of the respective nucleotide in 50 mM Hepes, pH 7.3, 1 mM magnesium acetate, 10 mM EDTA, 2.5 mM DTT, 1 mM ATP. After 2.5-fold dilution and addition of magnesium acetate to 5 mM, free nucleotides were removed by gel filtration on Superose 12. When Ran was loaded with radioactive nucleotides, only 25  $\mu\text{M}$  unlabeled GTP was added together with the radioactive nucleotide (10–50  $\mu\text{Ci}/100 \mu\text{l}$  reaction). In this case, nonincorporated nucleotides were removed by gel filtration over disposable Nap5 columns (Pharmacia).

## Ran Binding to Rat Liver Nuclei or Nuclear Envelopes

Rat liver nuclei and NEs were isolated as described (Snow et al., 1987). Nuclei at a concentration of 25 OD<sub>260</sub>/ml were incubated with 1  $\mu\text{M}$  Ran preloaded with either GMP-PNP or GDP for 20 min at 30°C. After fixation with 3.7% formaldehyde in PBS, the nuclei were bound to poly-L-lysine-coated coverslips and Ran was visualized by indirect immunofluorescence microscopy. Purified rat liver NEs at a concentration of 100 OD<sub>260</sub>/ml were incubated without Ran or with 2  $\mu\text{M}$  Ran preloaded with either GMP-PNP or GDP for 20 min at 30°C. After fixation with 3.7% formaldehyde in PBS for 10 min at RT, the envelopes were incubated in

suspension for 1 h at RT with  $\alpha$ -Ran antibodies in PBS and 2% BSA, washed, and subsequently incubated for 2 h at RT with goat  $\alpha$ -rabbit antibodies conjugated to 5 nm colloidal gold particles. The cells were then fixed with 2% glutaraldehyde in PBS containing 0.02% tannic acid and post-fixed with 1% osmium tetroxide, dehydrated, and embedded for electron microscopy. Thin sections were stained with 2% uranyl acetate for 1 min.

## Identification of Ran-GTP-binding Proteins in Nuclear Envelopes by Blot Overlay

10 OD<sub>260</sub> U NE per lane were separated by SDS-PAGE and transferred to nitrocellulose. After incubation of the membranes in PBS, 0.05% Tween 20, and 0.5% BSA for 30 min at RT the membranes were incubated for 30 min at RT with 10 nM labeled Ran (see above) in PBS, 0.05% Tween 20, 0.5% BSA, 2 mM DTT, and 50  $\mu\text{M}$  GTP. After three brief washes in binding buffer the filters were dried and analyzed either using a PhosphorImager (Molecular Dynamics, Sunnyvale, CA) or by autoradiography. For immunoprecipitation, envelopes were first solubilized at 200 OD<sub>260</sub>U/ml in 20 mM Tris, pH 8.8, 1% Triton X-100, 500 mM NaCl, 1 mM EDTA, 2 mM DTT, 1 mM PMSF, and 1  $\mu\text{g/ml}$  each of leupeptin, pepstatin, and aprotinin for 2 h on ice. After a 100,000 g spin for 30 min the supernatant was incubated with antibodies overnight on ice. Immunoabsorbed proteins were recovered by incubation with protein A Trisacryl beads (Pierce) for 3 h, washed two times in solubilization buffer and extracted from the beads in SDS-PAGE loading buffer. To control for nonspecific binding, the same procedure omitting the antibodies was carried out in parallel.

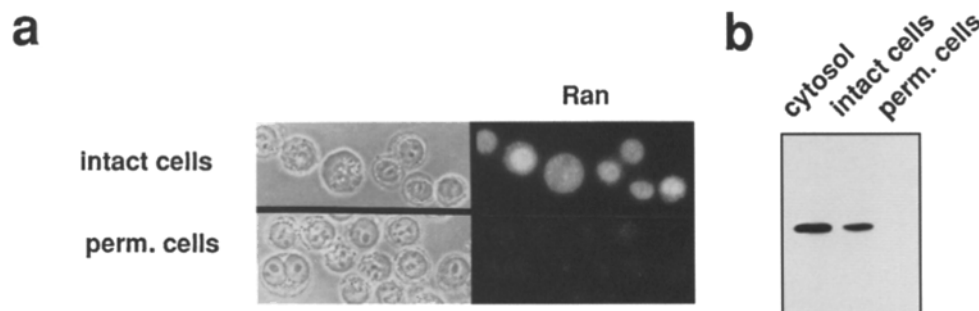
## Gel Electrophoresis and Immunoblotting

For SDS-PAGE and Western transfer standard protocols were followed. Detection of Ran/TC4 on immunoblots was performed as follows: membranes after transfer were saturated with 5% milk powder in PBS and 0.2% Tween 20 for 1 h at RT, incubated with  $\alpha$ -Ran antibodies at a dilution of 2  $\mu\text{g/ml}$  in PBS, 0.05% Tween 20, and 2% milk powder for 1 h at RT, washed twice for 10 min with 0.2% Tween 20 in PBS, incubated for 1 h with peroxidase-conjugated  $\alpha$ -rabbit IgGs (1:5,000) in PBS, 0.05% Tween 20, and 2% milk powder, and washed as before. Detection of antibodies was carried out using the ECL detection system (Amersham Corp., Arlington Heights, IL).

## Results

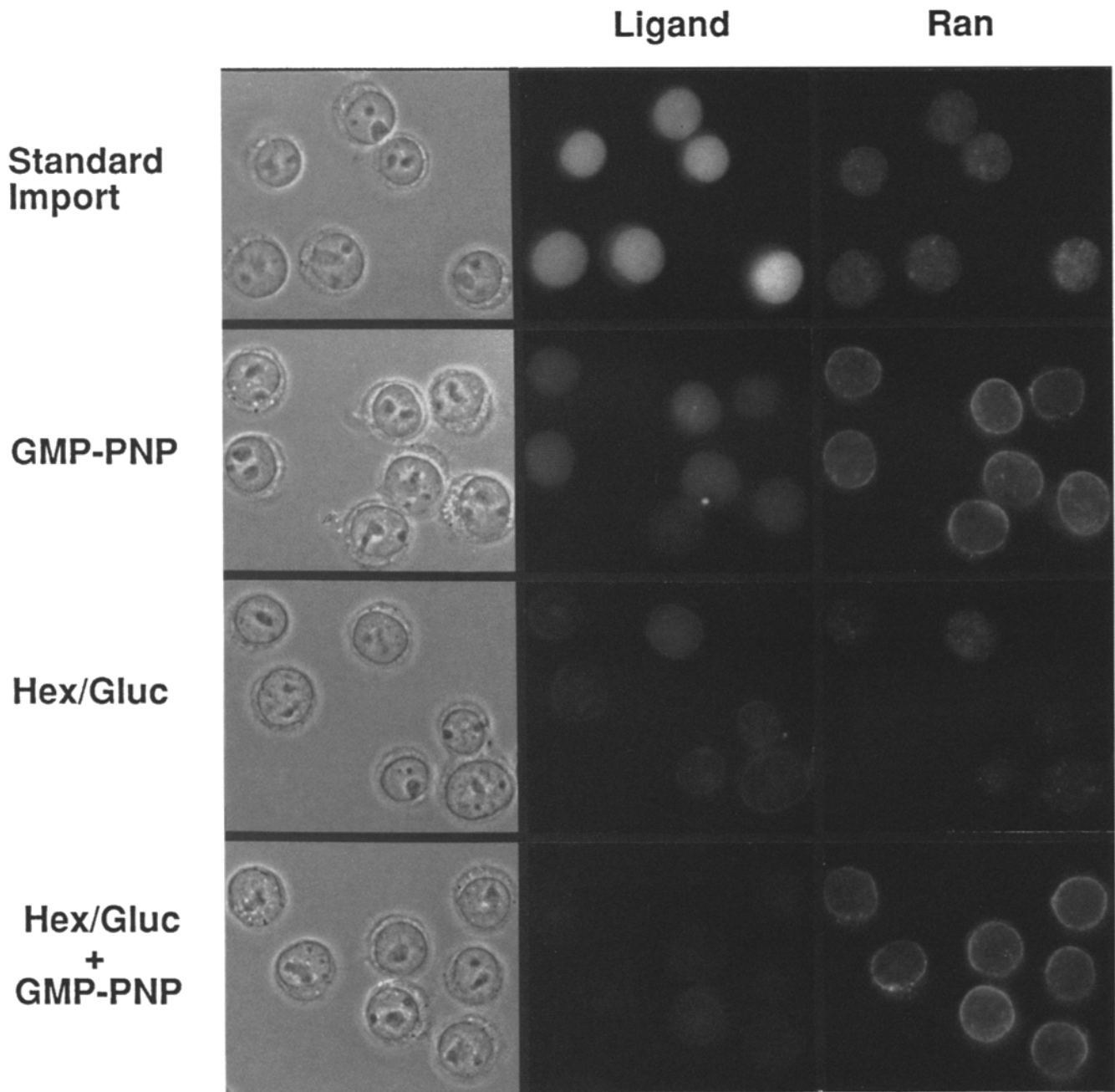
### Localization of Ran in Permeabilized Cells under Conditions of Nuclear Transport Arrest by Nonhydrolyzable GTP Analogs or ATP Depletion

To characterize the localization of Ran in permeabilized cells under different transport conditions, we prepared affinity purified antibodies against the COOH-terminal hypervariable region of this protein (similar to Ren et al.,



**Figure 1.** Digitonin permeabilization of HeLa cells leads to rapid release of Ran. (a) Detection of Ran in intact and digitonin permeabilized cells by immunofluorescence microscopy. HeLa suspension cells were either fixed with formaldehyde directly (*intact cells*) or permeabilized with digitonin and washed as described for the in vitro nuclear import assay

prior to fixation (*perm. cells*). The cells were mounted on coverslips, permeabilized with Triton X-100, and probed for Ran. (b) Detection of Ran by immunoblot analysis in intact and digitonin permeabilized cells and in cytosol used for transport assays. 50  $\mu\text{g}$  cytosolic proteins (*cytosol*),  $2 \times 10^5$  intact cells (*intact cells*) and  $2 \times 10^5$  digitonin permeabilized cells (*perm. cells*) were separated by SDS-PAGE on a 12.5% gel, transferred to nitrocellulose and probed for Ran. The relative amounts of cytoplasm and permeabilized cells analyzed in lanes 1 and 3 are the same as used in the in vitro transport assays.



**Figure 2.** The nonhydrolyzable GTP analog GMP-PNP leads to accumulation of Ran but not ligand at the nuclear envelope and is dominant over the effect of ATP depletion. Transport reactions were carried out under standard conditions (*Standard Import*), or in the presence of 200  $\mu$ M GMP-PNP (*GMP-PNP*); 16 U hexokinase/5 mM glucose (*Hex/Glu*); or GMP-PNP and hexokinase/glucose (*Hex/Gluc + GMP-PNP*). After transport the cells were fixed and permeabilized, and Ran was localized by immunofluorescence microscopy (*right panels*). The accumulation of fluorescent transport ligand is shown in the middle panels, and phase contrast images are shown in the left panels.

1993). With these antibodies, we found that the localization of Ran in intact HeLa cells was predominantly nuclear (Fig. 1 *a*) as reported previously (Ren et al., 1993). By contrast, both immunofluorescence microscopy (Fig. 1 *a*) and immunoblotting (Fig. 1 *b*) indicated that most Ran was depleted from HeLa suspension cells during digitonin treatment and released into the cytosol (compare *intact cells with perm. cells*, Fig. 1 *b*). From quantitative immunoblotting, more than 90% of Ran in intact cells was released

during cell permeabilization (data not shown). Thus in the permeabilized cell transport assays Ran is provided almost exclusively by the exogenous cytosol (see Fig. 1 *b* legend).

We previously found that inhibition of *in vitro* nuclear import by nonhydrolyzable GTP analogs is mediated at least predominantly by Ran (Melchior et al., 1993 *a*). Under these conditions Ran should be locked in its “activated” form and therefore might accumulate at the site where Ran normally hydrolyzes GTP. To examine this, we

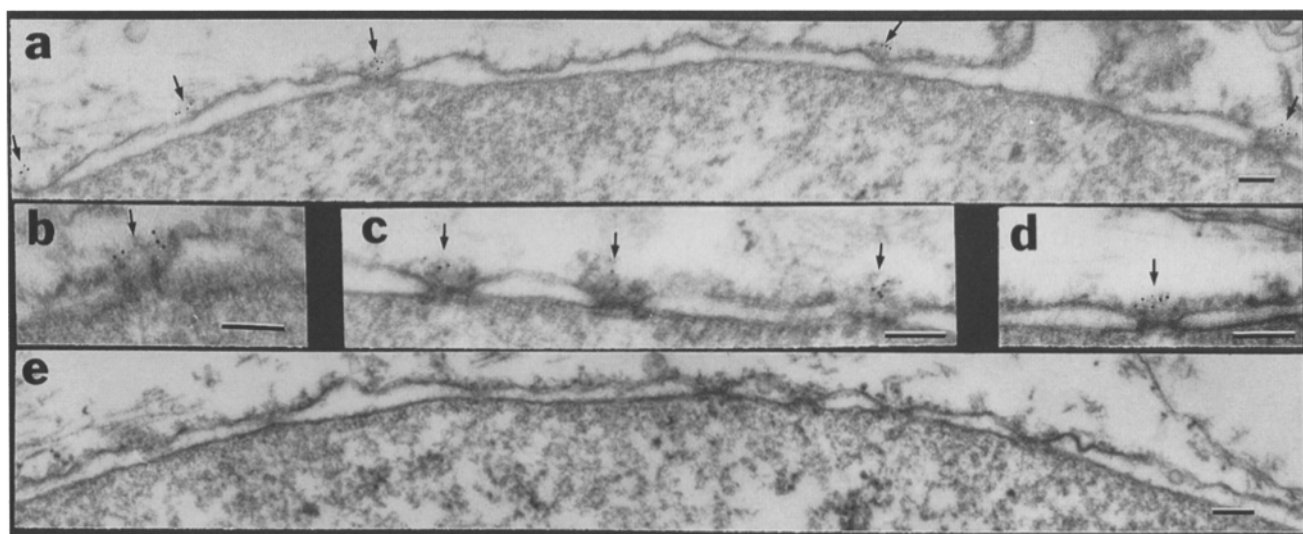
used light microscopy to characterize the localization of Ran and transport ligand in permeabilized cell assays in the presence or absence of the nonhydrolyzable GTP analog GMP-PNP (Fig. 2, *Standard Import, GMP-PNP*). To relate these findings to the ATP-independent step for ligand binding at the NPC, we examined Ran and transport ligand localization in assays where transport was inhibited by depletion of ATP with hexokinase/glucose (Fig. 2, *Hex/Gluc*), and in assays containing both hexokinase/glucose and GMP-PNP (Fig. 2, *Hex/Gluc + GMP-PNP*). In standard transport assays, the only Ran that was found was intranuclear. Strikingly, addition of GMP-PNP led to strong accumulation of Ran at the NE, as indicated by the nuclear rimlike staining pattern. Similar results were obtained using the nonhydrolyzable GTP analog GTP- $\gamma$ -S instead of GMP-PNP (data not shown). Omission of the Triton permeabilization of fixed cells prior to incubation with antibodies for immunolocalization did not eliminate the Ran staining (data not shown), indicating that at least part of it was due to association of Ran with the cytoplasmic side of the NE, which, unlike the nucleoplasmic side of the NE, is directly accessible to antibodies in digitonin-permeabilized cells (Adam et al., 1990; see also Fig. 3).

As described previously (Newmeyer and Forbes, 1988; Richardson et al., 1988), depletion of ATP from the nuclear import assays led to the accumulation of NLS-containing transport ligand at the NE (Fig. 2, *Hex/Gluc*). However, this condition did not cause Ran to become concentrated at the NE. Interestingly, the addition of GMP-PNP to assays where ATP was depleted (Fig. 2, *Hex/Gluc + GMP-PNP*) yielded results similar to the addition of GMP-PNP alone, i.e., Ran accumulated at the NE and either no ligand or only a low level of ligand accumulated at the NE, depending on the cytosol preparation used. This could imply that a transport step requiring GTP hydrolysis precedes the first step requiring ATP. However, a body of

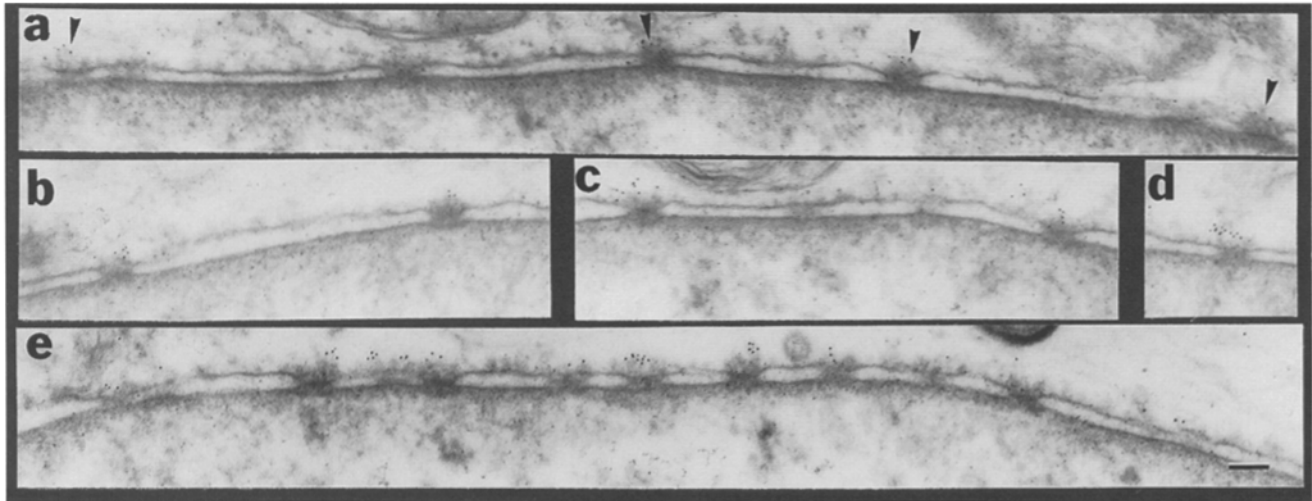
other data argue that both the GMP-PNP treatment and the ATP depletion affect the same transport step (see Discussion), but with different outcomes depending on whether Ran is in the GTP form (in the presence of GMP-PNP) or in the GDP form (occurring with ATP depletion).

We next used immunoelectron microscopy to investigate whether the nuclear rim accumulation of Ran in the presence of GMP-PNP reflected binding of Ran to NPCs (Fig. 3). To preserve cellular membranes in these samples, cells were not permeabilized with Triton prior to the immunolabeling, and therefore only Ran on the cytoplasmic surface of the NPC was accessible for detection. While very little Ran was detected at the NE in a control reaction lacking GMP-PNP (Fig. 3 *e*), strong accumulation of Ran at the cytoplasmic surface of the NPC was found in the assays containing GMP-PNP (Fig. 3, *a-d*, arrows). Individual NPCs were often labeled with multiple gold particles, and most of the labeling was found in the peripheral region of the NPC.

The accumulation of Ran at the periphery of the NPC in the presence of GMP-PNP suggests that Ran could be binding to the fibrils that emanate from the cytoplasmic surface of the NPC (Jarnik and Aebi, 1991). These fibrils are thought to be the site where ligands initially bind at the NPC during nuclear import (Richardson et al., 1988). Since depletion of ATP arrests transport ligand at peripheral binding sites in the NPC that may correspond to the cytoplasmic fibrils (Newmeyer and Forbes, 1988; Richardson et al., 1988), we analyzed the localization of NLS-containing transport ligand at the NPC by immunogold electron microscopy in permeabilized cells where ATP was depleted (Fig. 4). We found that under this condition NLS-containing ligand accumulated at a peripheral region of the NPC that was very similar to the area of Ran accumulation in the presence of GMP-PNP (compare Fig. 4, *a-d* with *e*).



**Figure 3.** Ran accumulates at the cytoplasmic periphery of the nuclear pore complex in the presence of the nonhydrolyzable GTP analog GMP-PNP. Shown are thin section electron micrographs of the NE of permeabilized cells after transport in the presence of GMP-PNP (*a-d*) or under standard conditions (*e*). After transport cells were fixed, labeled with antibodies to detect Ran, and processed for electron microscopy (Materials and Methods). Thin sections were stained with 2% uranyl acetate. (Arrows) Gold clusters. Note that nuclear membranes were not permeabilized with Triton, precluding detection of intranuclear Ran. Bars, 100 nm.

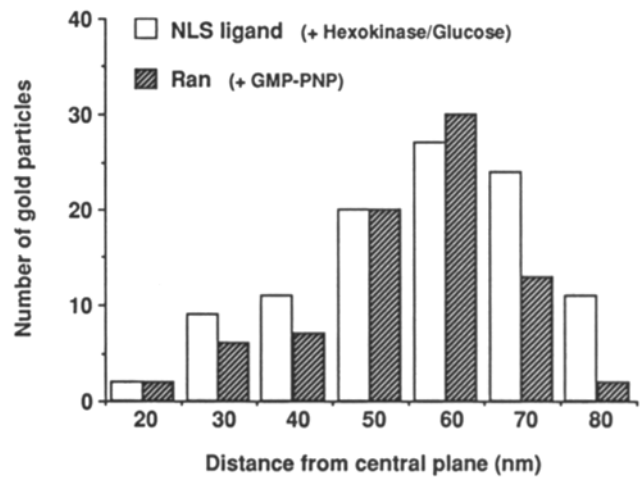


**Figure 4.** ATP depletion leads to accumulation of ligand at the cytoplasmic periphery of the nuclear pore complex. Shown are electron micrographs of the NE in thin sections of permeabilized cells after transport in the presence of hexokinase/glucose (a–d) or GMP-PNP (e). After transport cells were fixed, stained with antibodies to detect ligand (a–d) or Ran (e), and processed for electron microscopy (Materials and Methods). Thin sections were stained with 2% uranyl acetate. (Arrows) Gold clusters. Note that nuclear membranes were not permeabilized with Triton, precluding the detection of intranuclear Ran or ligand. Bars, 100 nm.

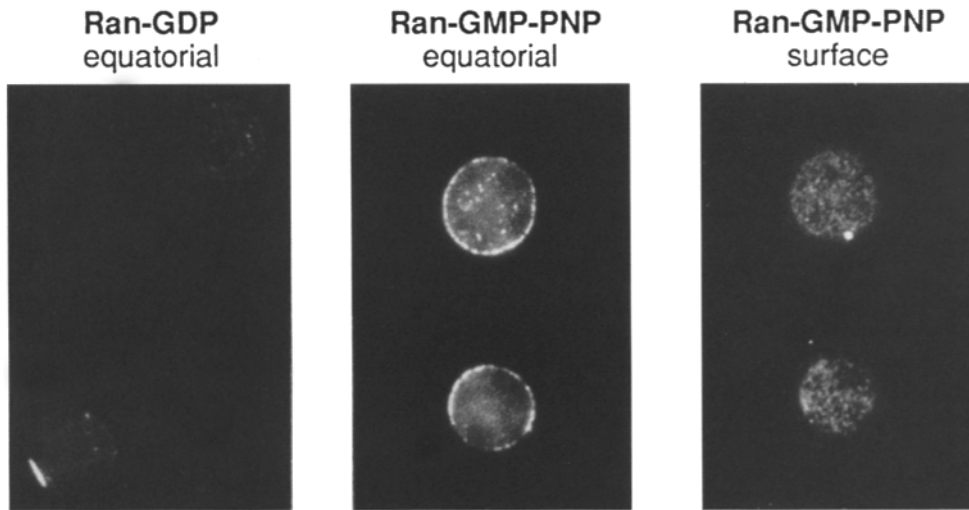
We quantitatively compared the localization of Ran in assays containing GMP-PNP with the localization of transport ligand in assays where ATP was depleted (Fig. 5). From this we determined that both Ran and ligand accumulated at a peak distance of 50–60 nm from the central plane of the NPC. A slightly higher number of gold particles was found for ligand compared to Ran at distances greater than 60 nm, though this may be due to differences in the accessibility of antibodies to Ran and ligand. Thus, within the resolution afforded by immunogold EM, the NPC region where Ran accumulates in the presence of GMP-PNP is indistinguishable from the area of ligand binding under ATP depletion conditions. This localization reflects the region occupied by the cytoplasmic fibrils implicated in initial ligand binding at the NPC, and is more peripheral than the cytoplasmic “ring” of the NPC (located about 40 nm from the central plane of the NPC; Hinshaw et al., 1992). Furthermore, the region of Ran and ligand accumulation is considerably more peripheral than the O-linked glycoprotein p62, which is localized 20–30 nm from the central plane of the NPC (Guan, et al., 1995).

The similarity between the NPC region where Ran accumulates when GTP hydrolysis is inhibited, and the area where transport ligand associates when ATP is depleted, is consistent with the model that GTP hydrolysis by Ran plays a role at the site of initial ligand binding. It seems likely that the GMP-PNP-bound form of Ran accumulates at the NPC when transport is inhibited, since the inhibitory effects of the nonhydrolyzable GTP analogs are at least predominantly mediated by Ran (Melchior et al., 1993a). To directly examine this question, we replaced the endogenous Ran in the cytosol fraction of nuclear import assays with Ran preloaded with GMP-PNP. However, no transport inhibition was obtained in this case, presumably due to rapid exchange of the nonhydrolyzable analog on Ran with GTP after addition to the transport assay, either by intranuclear RCC1 (Ohtsubo et al., 1989) or RCC1

present in our cytosol fraction (data not shown). While this experiment was unsuccessful, we demonstrated an interaction of Ran-GMP-PNP with the NPC by direct binding experiments with isolated NEs (see below). Moreover, a mutant of Ran that is unable to hydrolyze GTP (Ran[Q69L]; Klebe et al., 1995) accumulates at the nuclear envelope when added to the permeabilized cell assay and leads to inhibition of import (Melchior, F., J. Becker,



**Figure 5.** Both Ran and transport ligand accumulate at similar distances from the central plane of the NPC when transport is arrested by GMP-PNP and hexokinase/glucose, respectively. EM pictures from the experiment shown in Fig. 4 were used to measure the position of gold particles relative to the central plane of the NPC. (White bars) Distribution of gold particles after transport in the presence of hexokinase/glucose and antibody staining for the ligand; (Striped bars) distribution of gold particles after transport in the presence of GMP-PNP and antibody staining for Ran.



**Figure 6.** Ran-GMP-PNP, but not Ran-GDP binds directly to isolated rat liver nuclei. Nuclei at a concentration of 25 OD<sub>260</sub>/ml were incubated with 1  $\mu$ M Ran preloaded with either GMP-PNP (b and c) or GDP (a) for 20 min at 30°C, fixed with formaldehyde, mounted onto coverslips, and probed for Ran by immunofluorescence microscopy.

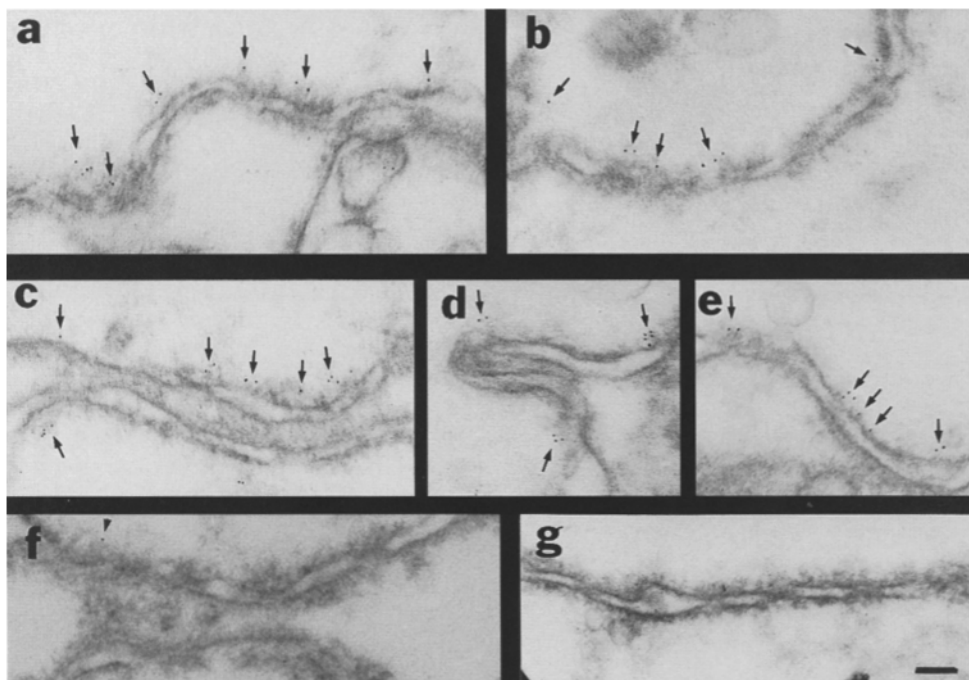
L. Gerace, unpublished results), similar to wild type Ran in the presence of nonhydrolyzable GTP analogs.

#### **Direct Interaction of Ran-GTP with the Nuclear Pore Complex**

The accumulation of Ran at the NPC in permeabilized cell transport assays containing GMP-PNP could reflect a direct interaction of Ran with an NPC protein or an indirect interaction of Ran with the NPC via a cytosolic linker. To distinguish between these possibilities, we investigated

whether recombinant Ran was able to bind to isolated rat liver nuclei in the absence of other cytosolic components. Using immunofluorescence microscopy, we found that Ran preloaded with GMP-PNP strongly bound to isolated nuclei, while Ran preloaded with GDP showed almost no interaction (Fig. 6). In nuclei incubated with Ran-GMP-PNP, Ran immunolocalization yielded nuclear rim staining in an equatorial view and punctate labeling in a surface view, consistent with an NPC localization.

We next examined the binding of recombinant Ran to isolated rat liver NEs using immunogold electron micros-



**Figure 7.** Ran-GMP-PNP binds specifically to the cytoplasmic side of isolated rat liver nuclear envelopes. Shown are thin section electron micrographs of isolated rat liver nuclear envelopes after incubation with Ran-GMP-PNP (a-e), Ran-GDP (f), or without Ran (g). Rat liver NEs at a concentration of 100 OD<sub>260</sub>U/ml were incubated without Ran or with 2  $\mu$ M Ran preloaded with either GMP-PNP or GDP for 20 min at 30°C, fixed with formaldehyde, incubated with  $\alpha$ -Ran antibodies, and gold-conjugated secondary antibodies to detect Ran, and subsequently processed for electron microscopy. It was possible to unambiguously distinguish the inner and outer nuclear membranes in these isolated NEs (see Snow et al., 1987; Senior and Gerace, 1988) because the latter was frequently ruptured due to connections with peripheral ER (e.g., top NE shown in c). (Arrows) Gold clusters. Bar, 100 nm.

copy (Fig. 7). This work showed that Ran preloaded with GMP-PNP (Fig. 7, *a-e*) or GTP (not shown) but not with GDP (Fig. 7 *f*) bound strongly to NPCs in these preparations. Similar binding was obtained with NEs that were prewashed with 0.5 M NaCl prior to incubation with Ran to remove loosely bound components (data not shown). Interestingly, gold was found almost exclusively at the cytoplasmic surface of the NPC, even though in these experiments the nucleoplasmic surface was freely accessible. These data indicate that Ran-GMP-PNP binds directly to a component of the NPC in the absence of additional cytosolic factors, and that this binding site is restricted to the cytoplasmic surface of the NPC. Quantitative analysis showed that the peak of Ran labeling was about 55 nm from the central plane of the NPC (data not shown). Since this is very similar to the region of the NPC where Ran accumulates in transport assays containing GMP-PNP (see Figs. 3-5), this strongly supports the interpretation that Ran-GMP-PNP accumulates close to the initial ligand binding sites when transport is inhibited.

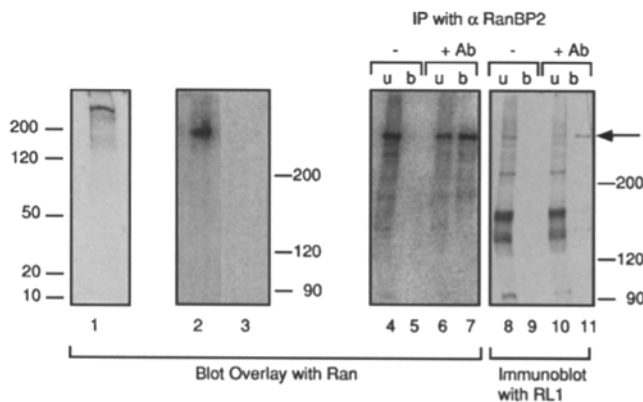
### Identification of RanBP2 as the Predominant Ran-GTP-binding Protein of Isolated Nuclear Envelopes

To identify putative binding partners for Ran-GTP in the NPC, we examined isolated NEs for the presence of proteins that bind Ran-GTP in an SDS gel blot overlay assay. This technique has been successful in identifying the 24-kD Ran-binding protein RanBP1 (Coutavas et al., 1993; Bischoff et al., 1995*b*) as well as several other candidate in-

teracting proteins in cultured cells and tissues (Lounsbury et al., 1994). We found a prominent ~350-kD band in rat liver NEs that specifically bound Ran-GTP but not Ran-GDP (Fig. 8, lanes 1-3). In addition, we also detected a group of 90-350-kD bands that bound Ran-GTP more weakly and varied in intensity relative to the ~350-kD band between different experiments. The ~350-kD band behaves as a peripheral membrane protein that is tightly associated with the NE, since little of it is solubilized by treatment with 2 M NaCl, whereas most of it is extracted by treatment with 8 M urea (data not shown).

A 358-kD Ran-binding protein (RanBP2) with homology to nuclear pore complex proteins had recently been identified by two hybrid interaction cloning (Yokoyama et al., 1995) and it seemed likely that the major protein we detected in rat liver NEs was a homolog of RanBP2. To investigate this, we used affinity-purified  $\alpha$ -peptide antibodies against human RanBP2 to immunoprecipitate the rat protein from solubilized NEs (Fig. 8, lanes 4-7). The ~350-kD protein as well as most of the faster migrating bands detected by blot overlay were specifically immunoadsorbed by the antibodies, indicating that this protein is indeed the rat liver homolog of RanBP2. Since most of the smaller polypeptides appear to be degradation products of RanBP2, it seems that RanBP2 is the only prominent Ran-GTP-binding protein in nuclear envelopes. However, the presence of other Ran binding proteins not detectable by this overlay technique cannot be ruled out.

RanBP2 contains multiple FG repeat motifs characteristic of a group of NPC proteins that are modified with O-linked *N*-acetyl glucosamine (reviewed in Rout and Wente, 1994) and recognized by the monoclonal antibody RL1 (Snow et al., 1987). Since the reactivity of the antibody depends on the presence of the sugar modification (Holt et al., 1987), it could be used to determine whether RanBP2 belongs to this family of proteins. We therefore probed a RanBP2 immunoprecipitate with RL1 antibody (Fig. 8, lanes 8-11) and found that RanBP2 is indeed an RL1 antigen and thus is predicted to contain O-linked *N*-acetyl glucosamine. However, attempts to bind rat liver RanBP2 to wheat germ agglutinin (a lectin which binds to *N*-acetyl glucosamine) or immunoprecipitation with RL1 have not been successful, which may indicate a relatively low level of sugar modification that precludes multivalent binding by lectin or antibody.



**Figure 8.** Ran-BP2 is the major Ran-GTP binding protein of isolated rat liver nuclear envelopes and a member of the RL1 family of O-linked glycoproteins. Lanes 1-7 (*Blot Overlay with Ran*): NEs (lanes 1-3) and the unbound (*u*; lanes 4 and 6) and bound (*b*; lanes 5 and 7) fraction of solubilized nuclear envelopes after immunoprecipitation with  $\alpha$ -RanBP2 antibodies (lanes 6 and 7) or control precipitation with protein A beads only (lanes 4 and 5) were analyzed by incubating SDS gel transfers with Ran preloaded with appropriate nucleotides. (Lane 1) 5-15% gradient gel probed with  $^{35}\text{S}$ -labeled Ran-GTP $\gamma$ S; (lane 3) 5% gel probed with  $^{32}\text{P}$ -labeled Ran-GDP; (lanes 2, and 4-7): 5% gel probed with  $^{32}\text{P}$ -labeled Ran-GTP. Data were collected either by autoradiography (lane 1) or using a phosphorimager (lanes 2-7). The background caused by Ran-GDP was usually lower than for Ran-GTP. (Lanes 8-11) after the overlay, lanes 4-7 were probed with the monoclonal antibody RL1.

### Discussion

GTPases function as molecular switches in diverse cellular processes (Bourne et al., 1990). They cycle between an "active" GTP-bound conformation and an "inactive" GDP-bound conformation by hydrolysis of GTP to GDP and subsequent exchange of GDP for GTP. Analogs of GTP such as GMP-PNP or GTP $\gamma$ S are resistant to hydrolysis and therefore lock GTPases in their active conformation. These analogs have been used in many systems to identify and analyze the function of GTPases, and led us to the identification of Ran as an essential transport factor (Melchior et al., 1993*a*). In the present study we have used non-hydrolyzable GTP analogs to investigate the step of transport that involves hydrolysis of GTP by Ran.

A key observation from this work was the finding that



inhibition of nuclear import by GMP-PNP leads to accumulation of Ran at peripheral cytoplasmic regions of the NPC. Ran appears to be reversibly bound to the NPC under these conditions, since transport inhibition by GMP-PNP can be reversed by addition of fresh cytosol to cells that have been preincubated with cytosol and GMP-PNP, or more simply, by addition of an excess of GTP to these assays (Melchior, F., and L. Gerace, unpublished results). Direct binding experiments involving recombinant Ran and isolated NEs showed that Ran preloaded with GTP or with GMP-PNP but not with GDP binds to the same peripheral cytoplasmic region of the NPC where Ran accumulates in permeabilized cell assays incubated with GMP-PNP. These data indicate that the GMP-PNP form of Ran is the species that accumulates at the cytoplasmic surface of the NPC when transport assays are incubated in the presence of the inhibitor. Moreover, these data argue that the association of Ran with the NPC in transport assays is direct and not dependent on other cytosolic factors. In combination with our earlier data showing that Ran mediates inhibition of transport by nonhydrolyzable GTP analogs (Melchior et al., 1993a) these results indicate that inhibition of transport by GMP-PNP is a consequence of the binding of Ran-GMP-PNP to the cytoplasmic periphery of the NPC, and that this is the site where Ran normally hydrolyzes its GTP.

In the presence of GMP-PNP, we found that Ran accumulates on the cytoplasmic side of the NPC at a peak distance of ~60 nm from the central plane. This site of Ran accumulation corresponds to the region occupied by the fibrils that emanate from the cytoplasmic surface of the NPC (Jarnik and Aebi, 1991), which are suggested to be the sites of initial ligand binding to the NPC during nuclear import (Richardson et al., 1988). Moreover, we found that this Ran binding region is essentially coincident with the region where NLS ligands accumulate at the NPC when nuclear import is inhibited by ATP depletion, which is presumed to arrest transport at the initial NPC binding step. Therefore, these data support the notion that GTP hydrolysis by Ran is closely linked to initial ligand binding to the NPC (see below). We cannot rule out the possibility that nonhydrolyzable GTP analogs also inhibit additional hypothetical GTPases required for protein import. However, these would likely act downstream of Ran function, since ligand binding to the NPC in the absence of Ran is insensitive to GMP-PNP (Moore and Blobel, 1993).

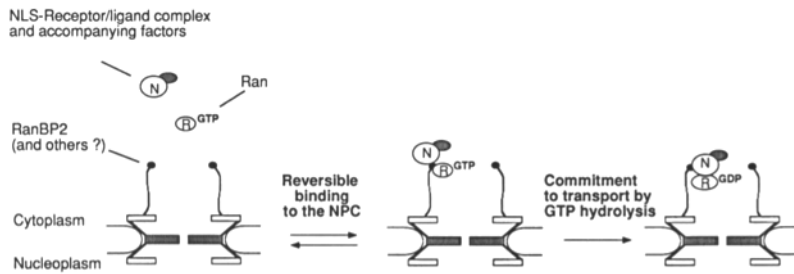
A simple interpretation of our findings related to Ran and ligand binding to the NPC would be that hydrolysis of GTP by Ran is required for targeting ligand to this site. However, this is unlikely to be correct, since Ran does not seem to be required for the initial binding of ligand to the NPC (Moore and Blobel, 1993; Adam and Adam, 1994; Moroianu et al., 1995; Görlich et al., 1995) and addition of Ran and GTP releases transport ligand from the binding sites occupied under conditions of ATP depletion (Moore and Blobel, 1994a). An alternative model in which hydrolysis of GTP by Ran is required immediately downstream of ligand binding is consistent with both our data and the studies mentioned above, if the effect of ATP depletion on GTP levels is taken into account. Endogenous nucleoside diphosphokinases rapidly interconvert these two nucleotides, so addition of hexokinase/glucose leads to ATP as

well as GTP depletion and consequently to depletion of Ran-GTP. If hydrolysis of Ran-GTP is required for progression of ligand from its initial binding sites at the NPC, depletion of Ran-GTP would lead to stable accumulation of ligand at peripheral sites. Since Ran-GMP-PNP can still bind to the NPC but cannot hydrolyze its bound nucleotide, it might destabilize bound ligand and thus prevent accumulation both in the presence or absence of ATP. Therefore, the transport block that is detected in permeabilized cells under conditions of ATP depletion most likely stems from GTP rather than ATP depletion, although later steps (e.g., central channel gating) presumably require ATP.

The binding site for Ran-GTP at the cytoplasmic side of the NPC is likely to be RanBP2/Nup358, a 358-kD Ran-GTP binding protein that has recently been identified (Wu et al., 1995; Yokoyama et al., 1995). Several lines of evidence support this hypothesis. First, we have shown that Ran-GTP binds to the cytoplasmic periphery of the NPC at a distance from the central plane of the NPC that is similar to the region of the NPC where RanBP2 has been localized by immunogold labeling (Wu et al., 1995; Yokoyama et al., 1995). Furthermore, we found that RanBP2 is the only Ran-GTP binding protein in isolated rat liver NEs that is detectable by blot overlays. Finally, we found that RanBP2 is a member of the RL1-reactive family of O-linked glycoproteins of the NPC previously implicated in nuclear transport and shown to interact with cytosolic transport factors (Sterne-Marr et al., 1992; Paschal and Gerace, 1995; Radu et al., 1995). Furthermore, an essential role for RanBP2 in import has been strongly suggested by the finding that antibodies to RanBP2 inhibit nuclear protein import *in vitro* (Yokoyama et al., 1995). However, unequivocal demonstration that RanBP2 provides the binding site for Ran in protein import will require further studies, such as chemical cross linking after *in vitro* transport.

The fate of Ran after hydrolysis remains to be analyzed in detail, but since the only known guanine nucleotide exchange factor for Ran, RCC1, is bound to chromatin (Ohtsubo et al., 1989) and since immunodepletion of RCC1 from *Xenopus* egg extracts removes nearly all detectable exchange activity for Ran (Dasso, 1994), it appears likely that the reformation of Ran-GTP by nucleotide exchange takes place in the nucleus. In one possible scenario, Ran-GDP would bind to the receptor/ligand complex and be cotransported into the nucleus. Nucleotide exchange might then occur only after disassembly of the transport complex, or might itself be the cause of complex disassembly. Alternatively Ran-GDP could dissociate from the transport complex prior to its transport through the gated channel and subsequently enter the nucleus by diffusion.

The data discussed above suggest a working model of the role of Ran-GTP in nuclear protein import, where Ran functions at the NPC site of initial ligand binding to commit the transport ligand to more distal steps of the nuclear import pathway (Fig. 9). We propose the existence of two distinct but nearby binding sites at cytoplasmic fibrils of the NPC that allow reversible binding of Ran and the NLS ligand/receptor complex. These binding sites are likely to be provided by RanBP2, possibly with additional components. When both Ran-GTP and the ligand/receptor



**Figure 9.** Working model for Ran function in an early step of nuclear protein import. This scheme proposes the receptor/ligand complex and Ran-GTP bind reversibly and independently to cytoplasmic fibrils of the NPC. After both sites are occupied, Ran hydrolyzes GTP, and Ran-GDP becomes part of the ligand/receptor complex. The hydrolysis step is envisioned to commit the ligand/receptor complex to transport.

complex have bound and are able to interact, GTP is hydrolyzed, Ran-GDP associates with the ligand/receptor complex and the newly formed import complex can engage in subsequent steps of transport. If hydrolysis of GTP by Ran does not occur in a timely fashion, such as when Ran is bound to nonhydrolyzable GTP analogs, transport would be aborted and the receptor/ligand complex would be induced to dissociate from the initial docking site while Ran remains bound. In the absence of Ran-GTP, such as could occur when ATP is depleted (see above) or when ligand becomes bound at the NE in permeabilized cell assays depleted of Ran, the receptor ligand complex would remain at the initial binding site, but “uncommitted” to the nuclear import pathway.

This model implies the presence of a GTPase activating factor at the cytoplasmic side of the NPC. This activity could be an intrinsic property of the receptor/ligand/Ran-GTP complex formed at the initial docking site, or it could be provided by an NPC component or another soluble factor. An excellent candidate GAP for nuclear import is RanGAP1, which has recently been shown to be homologous to Rna1p (Bischoff et al., 1995a; Becker et al., 1995), a yeast protein previously implicated in RNA export and processing (Tragila et al., 1989). Defects in *S. cerevisiae* Rna1 lead to phenotypes nearly identical with phenotypes caused by defects in the *S. cerevisiae* RCC1 homolog Prp20 (Forrester et al., 1992), supporting the possibility that they affect the same cellular pathway. The intracellular localization of RanGAP1 is not known, but its yeast homologs are localized in the cytoplasm and possibly enriched at the NE (Hopper et al., 1990; Melchior et al., 1993b). As RanBP1 has been shown to modulate RanGAP1 activity, it could be involved in this transport step (Bischoff et al., 1995b). Alternatively, RanGAP1 and RanBP1 might play a role in regulating the level of Ran-GTP in the cell, which could regulate the overall rate of nuclear import (see below). However, since it is not clear whether Ran solely functions in nuclear protein import or also is directly involved in other pathways, it is possible that neither of these factors is directly involved in import.

The location of GTP hydrolysis by Ran in this model positions Ran at a potentially key regulatory point in the nuclear import pathway. By acting at the peripheral region where ligand first associates with the NPC, Ran GTP hydrolysis could help to confer vectoriality on the nuclear import pathway. In addition, if the cellular concentration of Ran-GTP becomes limiting for nuclear import, Ran would be ideally positioned to regulate the overall rate of nuclear import, which seems to vary under different physiological conditions (Feldherr and Akin, 1994). It is noteworthy that Ran-GTP binds only to the cytoplasmic side

of the NPC. This is consistent with the notion that the only nuclear trafficking pathway directly involving Ran is protein import, and suggests that inhibition of RNA or protein export caused by defects in the Ran system might be indirect consequences of inhibition of nuclear import.

In conclusion, the findings presented here provide a refined analysis of the role of Ran in import. In contrast to suggestions that hydrolysis of GTP by Ran occurs at a late transport step after translocation into the nucleus (Moore and Blobel, 1994b), the data presented here indicate that GTP hydrolysis by Ran is an early transport step that occurs at the site of initial ligand binding. We propose that the hydrolysis of GTP by Ran provides the essential function of committing ligands to the nuclear import pathway and conferring vectoriality on this process.

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