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Mechanism of Nuclear Protein Import and Export an In Vitro Approach

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Mechanism of nuclear protein import and export:

an *in vitro* approach

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"Das, wonach wir uns sehnen,

muss doch im Bereich unserer Kräfte stehen"

"What we desire, must be within our reach"

Karoline von Günderrode,

in "Kein Ort. Nirgends" by Christa Wolf

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Abbreviations

ATP	adenosine triphosphate
BSA	bovine serum albumin
cNLS	classical nuclear localization signal
DNA	deoxyribonucleic acid
EDTA	ethylenediamine tetraacetic acid
ER	endoplasmic reticulum
GDP	guanosine 5'-diphosphate
GFP	green fluorescent protein
GMP-PCP	guanylyl-(β1,γ-methylene) diphosphonate
GMP-PNP	5'-guanylyl imido-diphosphate
GST	glutathione S-transferase
GTP	guanosine 5'-triphosphate
Hepes	N-2-hydroxyethylpiperazine-N'-2-ethane sulphonic acid
IPTG	isopropyl-1-thio-β-D-galactopyranoside
Kap	karyopherin
LMB	leptomycin B

mRNA	messenger ribonucleic acid
NE	nuclear envelope
NES	nuclear export signal
NLS	nuclear localization signal
NPC	nuclear pore complex
Nup	nucleoporin
PBS	phosphate buffered saline
PCR	polymerase chain reaction
Pi	inorganic phosphate
Ran	Ras-related nuclear protein
RanGAP	Ran GTPase activating protein
RanGEF	Ran guanine nucleotide exchange factor
RNA	ribonucleic acid
RNP	ribonucleoparticle
RRE	Rev response element
SDS	sodium dodecyl sulfate
SDS-PAGE	SDS-polyacrylamide gel electrophoresis
TCA	trichloroacetic acid

Tris	Tris (hydroxymethyl) aminomethane
tRNA	transfer ribonucleic acid
RU	resonance unit
U snRNA	U small nuclear ribonucleic acid

Abstract

Protein import and export into and out of the cell nucleus requires a signal in the protein to be transported. Furthermore, several factors are involved in recognition of the signal, delivery of the protein to the nuclear pore complex (NPC) and its subsequent translocation across the NPC. We have biochemically analyzed the mechanism of nuclear protein import and export using all recombinant factors from *Saccharomyces cerevisiae*. Using solution binding assays, GTP hydrolysis and exchange assays, as well as surface plasmon resonance we have identified various interactions between different components of the transport machinery. We propose a model for the mechanism for nuclear protein import and export. Both nuclear protein import and export share certain features, i.e. docking of the transport cargo at the NPC via a karyopherin, release of the cargo/ karyopherin complex, and subsequent recycling of the transport factors for another round of function. However, although the proteins of the general transport machinery involved are the same for nuclear protein import and export, some transport factors play different roles in import and in export.

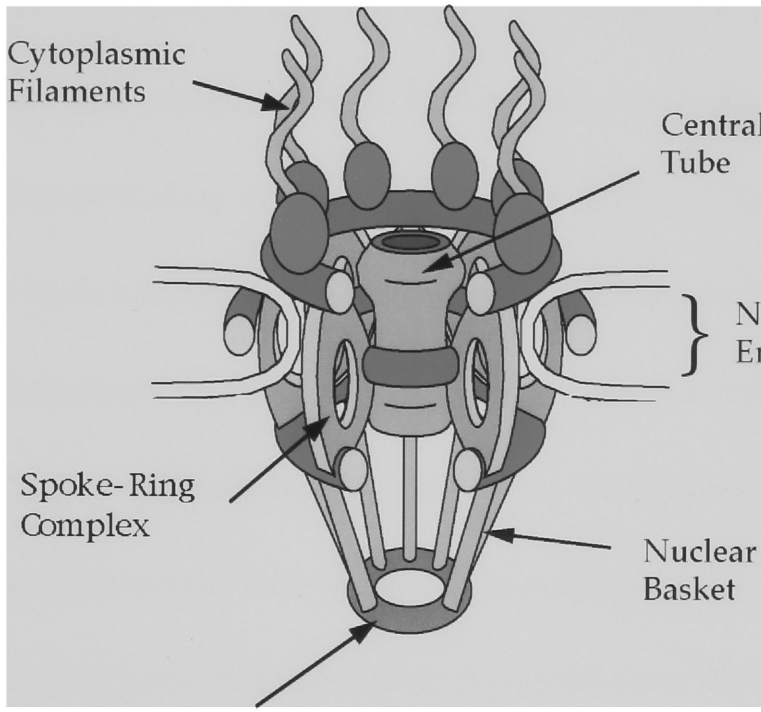
Chapter 1: Introduction to nuclear protein transport

1.1 Nuclear protein transport occurs through the nuclear pore complex

A major task an eukaryotic cell has to accomplish is the transport of macromolecules such as proteins, RNA and ribonucleoproteins (RNPs) in and out of the cell nucleus. The cell nucleus is surrounded by a double membrane, which is continuous with the endoplasmic reticulum (ER) and is called the nuclear envelope (NE). Large pores within the NE allow exchange of particles between the cytoplasm and the nucleoplasm. These pores were first identified as the sites of nucleocytoplasmic exchange by Feldherr in 1962 in studies investigating the intracellular movement of colloidal gold particles (1, 2). Later studies by Paine et al. of nucleocytoplasmic exchange of dextran molecules of different sizes suggested that the size limit for passive diffusion of particles through the nuclear pore is ~ 90 Å (3). The nuclear pore is a large proteinaceous channel and has been termed nuclear pore complex or NPC. The NPC has been extensively studied on a molecular and structural level (reviewed in (4, 5)), and a schematic view of the NPC is shown in Fig. 1 (Fig. 1 was kindly provided by M.P. Rout). An NPC from a mammalian cell has a molecular weight of 100-150 MDa and is thought to consist of 50-100 different proteins that have collectively been termed

Fig. 1 Schematic view of the NPC.

A description of the NPC structure is provided in the text.


Cytoplasmic
Filaments
Central
Tube
Nuclear
Envelope
Spoke-Ring
Complex
Nuclear
Basket
Terminal Ring

nucleoporins (nups). Structural studies using cryoelectronmicroscopy have revealed the highly symmetric structure of the central core of the NPC (6, 7). Eight spokes emanate from a central tube and are attached to an outer spoke ring that is embedded in the NE and extends into the NE lumen. The diameter of the NPC has been estimated to be ~ 120 nm, its length ~ 70 nm. The spokes are limited by ring like structures on the cytoplasmic and nucleoplasmic sides. Fibrils emanate from both the cytoplasmic and the nucleoplasmic rings, and contribute to a total length of the NPC along its longitudinal axis of ~ 200 nm from the tips of the cytoplasmic fibrils to the terminal ring of the nuclear basket like structure. The cytoplasmic fibrils are made up of different nucleoporins than the nucleoplasmic fibrils, which introduces asymmetry into the NPC architecture. This asymmetric distribution of nucleoporins on the cytoplasmic and nuclear side of the NPC might have interesting implications for directionality in nuclear transport. More than a dozen of nucleoporins have so far been identified from mammalian cells, and the identification of all NPC components from yeast is almost complete (Rout, M.P., personal communication). Interestingly, many nucleoporins contain certain peptide repeat motifs like FXFG, GLFG or XXFG (single letter amino acid code). Moreover, a number of these peptide repeat

containing nucleoporins have been localized to distinct sites in the NPC. This discrete localization of different nucleoporins might have interesting implications for nuclear transport and will be further discussed.

1.2 Nuclear import of endogenous proteins is signal dependent

First evidence that import of endogenous proteins into the nucleus occurs through the NPC was provided by microinjection experiments. In these experiments the nuclear protein nucleoplasmin from *Xenopus laevis* oocytes (8) or RN1 from *Rana pipiens* oocytes (9), that had been radioactively labeled, was injected into the cytoplasm of the respective oocytes and their translocation into the nucleus was followed. These studies indicated that the rate of nuclear import of RN1 was 20 times faster than what would be expected for passive diffusion through the nuclear pores (9). RN1 is a 150,000 mol-wt protein. Therefore, this finding suggested that import of large nuclear proteins into the nucleus is an active process. Further studies by Feldherr et al. of nuclear translocation of nucleoplasmin-coated gold particles revealed that particles up to a size of 200 Å could pass through the NPC (10). However, nuclear entry of these particles was dependent upon the presence of a nucleoplasmin coat, and import was abolished

when a carboxy-terminal tail region of nucleoplasmin was removed (8, 9). This result suggested that a signal in the carboxy-terminus of nucleoplasmin might be required for its import into the nucleus. Control experiments indicated that small gold particles (80-100 Å), which had been coated with polyvinylpyrrolidone (PVP), could enter the nucleus. However, import of these PVP-coated particles occurred at a much slower rate than import of particles of the same size that had been coated with nucleoplasmin. Interestingly, larger PVP-gold particles were not found in the nucleus after 1h reaction time. These results indicated that smaller particles might also enter the nucleus by passive diffusion, although signal dependent import is much faster. Import of larger molecules, however, was suggested to be completely dependent upon a signal. These investigators proposed that actively imported substances might contact specific sites inside the NPC, leaving some central space open that would allow for passive diffusion of smaller molecules. Alternatively, a gating mechanism was suggested according to which larger particles would trigger dilation of the NPC in a signal dependent manner (reviewed in (11)). However, evidence for this hypothetical gating mechanism remains elusive.

1.3 Identification of a nuclear localization signal

The hypothesis of a signal dependent import mechanism for nuclear proteins was further confirmed when short stretches of amino acids that are necessary and sufficient for nuclear localization were identified in various nuclear proteins (for review see (12)). Although there is no consensus sequence, it is now known that a short motif of mostly basic amino acids mediates nuclear import of a protein. Today, two classes of these classical nuclear localization signals or cNLSs have been identified. First, the SV-40 large T-antigen (PKKKRKV) like cNLSs that consist of a single stretch of 5-12 mostly basic amino acids. Second, there are bipartite cNLSs, which in addition to the first stretch of basic amino acids contain a second one in distinct spacing from the first. Such bipartite cNLSs have been found in nucleoplasmin (KRPAATKKAGQAKKKK) or in the human heat shock protein Hsp70 (FKRKHKKDISQNKRAVRR). In 1986 Goldfarb et al. found that nuclear import of BSA (bovine serum albumin), a protein of 66,000 mol-wt, conjugated with the cNLS from SV-40 large T-antigen showed saturation kinetics and occurred much faster than import of unconjugated BSA (13). Import of unconjugated BSA was linearly dependent upon its concentration indicating that it is driven only by diffusion. Furthermore, these investigators found that the

rate of import of cNLS-conjugated BSA decreased in the presence of free signal peptide, suggesting that free signal peptide competed for an important component of the import machinery. These findings led to the hypothesis of an import receptor that recognizes the cNLS (13). This receptor was proposed to be either localized at the NPC or, alternatively, to be freely diffusible in the cytoplasm (13).

1.4 The rate of nuclear protein import depends on the number of NLSs

Interestingly, it was shown by Dworetzky et al. that the rate of import depends upon the number of signal sequences (14). Using gold particles of different sizes that had been coated with cNLSs from the SV-40 large T-antigen these investigators found that an increase in the number of signals from 5 to 8 per particle increased import rates significantly. However, increasing the number of signals beyond 8 had no further effect on the rate of nuclear uptake. This correlation between import rates and the number of signals depended upon the size of the particles. Larger particles could be imported at the same rate as smaller particles when more signals were attached to them. However, the largest particles imported within the 1h reaction time had a diameter of 260 Å (including the cNLS coat), regardless of how many signals were attached. Together, these findings

suggested that within a certain size range the rate of import is dependent upon the number of signals, consistent with a receptor mediated import mechanism.

1.5 Nuclear protein import consists of two steps: docking of a cNLS-containing protein at the NPC, and translocation across the NPC

Additional evidence supporting the notion that import does not occur by simple diffusion but is an active process came from experiments that showed an energy requirement for nuclear import. Nuclear import of cNLS-conjugated HSA (human serum albumin) was found to be ATP and temperature dependent (15). Newmeyer and Forbes (16) as well as Richardson et al. (17) could further separate two steps in nuclear import: an ATP independent binding step of cNLS-conjugated HSA or nucleoplasmin at the cytoplasmic face of the NPC and subsequent ATP dependent translocation across the NPC. Using microinjection experiments these investigators found that a cNLS-containing protein could be docked at the cytoplasmic side of the nuclear pore in the absence of ATP, and that adding ATP to the system resulted in translocation of the docked cNLS-protein across the NPC. Furthermore, docking of a cNLS-containing protein on the cytoplasmic side of the NPC occurred at 4°C. However, translocation of the docked cNLS-protein

was only observed, when the temperature was shifted to 37°C. This reversible block of translocation, when the system was depleted of ATP or the temperature was decreased, suggested that translocation of a cNLS-protein across the NPC is an energy driven process. Newmeyer and Forbes also found that translocation but not the initial docking step was inhibited by the lectin wheat germ agglutinin (WGA)(16). Several nucleoporins were found to be glycosylated and to bind to WGA *in vitro* (18, 19) suggesting that WGA might block translocation by binding to specific sites in the NPC. WGA did not simply occlude the pore, since diffusion of small particles was not affected (18). One interpretation of these findings was that a cNLS-containing protein interacts first with nucleoporins on the cytoplasmic side of the NPC that do not bind WGA. During translocation, however, the cNLS-protein might interact with other nucleoporins that are sensitive to WGA (16). Interaction of the cNLS-protein with these nucleoporins would be inhibited, if WGA were bound to their sugar moiety.

Electronmicroscopic studies using nucleoplasmin-coated gold indicated that under conditions that prevented translocation nucleoplasmin-coated gold was associated with the cytoplasmic fibrils emanating from the NPC (17). This finding strongly suggested that initial docking of a cNLS-containing protein might occur on the

cytoplasmic fibrils.

1.6 Identification of different factors that mediate docking of a cNLS-containing protein at the NPC and subsequent translocation

Further insight into the docking and translocation steps on a molecular level came when Moore and Blobel found that these two processes require different cytosolic factors (20). For these studies Moore and Blobel used an *in vitro* system developed by Adam et al., where import of a fluorescently labeled cNLS-conjugate into the nucleus of digitonin-permeabilized cells is followed by fluorescence microscopy (21). Digitonin treatment permeabilizes the cell membrane while leaving the nuclear envelope intact. These investigators could show that a fraction of cytosol from *Xenopus laevis* oocytes, termed fraction A, was required for docking of cNLS-conjugated HSA at the NPC. A second cytosolic fraction, termed fraction B, stimulated translocation of the docked cNLS-conjugate when ATP was present. One component from fraction B was purified to homogeneity and identified as the Ras-related nuclear protein Ran (22, 23). Ran is a small GTPase of 25,000 mol-wt. Ran was originally cloned from a human teratocarcinoma cell line by Drivas et al. and called Ran/TC4 (24). Ran is a highly

abundant protein and has been estimated to represent 0.36 % of the total protein in HeLa cells (25). Unlike other small GTPases Ran is not posttranslationally modified, and therefore it is a soluble protein. Interestingly, 80 % of the cellular Ran is found in the nucleus (26). Ran was found to stimulate translocation of a cNLS-conjugate across the NPC. However, translocation was further stimulated, if small amounts of fraction B were present indicating that another factor in fraction B might be required for full translocation activity (22). Translocation of a cNLS-conjugate in the presence of Ran required the addition of free GTP. Initial experiments suggested that GTP hydrolysis was required for translocation (22). However, recent studies indicate that GTP hydrolysis on Ran might not be required for single turnover import, because import of a cNLS-conjugate also occurred in the presence of Ran and the non-hydrolyzable GTP analog GMP-PNP (27). Likewise, import was seen if a mutant Ran, RanQ69L, that cannot interact with the RanGTPase activating protein RanGAP was substituted for Ran. RanGAP stimulates the intrinsic GTPase activity of Ran by several orders of magnitude (28). These results suggested that neither intrinsic nor RanGAP mediated GTP hydrolysis on Ran is required for import. Nevertheless, the import rates seen in the presence of GMP-PNP or the RanQ69L mutant were lower than

those seen in the presence of wt Ran and GTP (27). We will present evidence that GTP hydrolysis is not required for any distinct step of nuclear import of a cNLS-protein but rather for recycling of Ran for another round of function.

1.7 The small GTPase Ran and its regulators

Ran had previously been isolated from HeLa cell nuclei in a complex with the chromatin associated protein Rcc1 (29). Rcc1 was shown to be a guanine nucleotide exchange factor for Ran (25), and will be termed RanGEF in the following. RanGEF has a homolog in *S. cerevisiae*, called Prp20 (30, 31). It has been estimated that only a small fraction of Ran in the nucleus is in complex with RanGEF, whereas the majority of Ran might be freely diffusible in the nucleoplasm (29). Ran, like all GTPases can exist in two forms: a GTP bound and a GDP bound form. Since RanGEF can exchange both GTP and GDP with the same efficiency, it is thought that the higher levels of GTP over GDP in the cell (30:1)(32) drive the exchange reaction in the direction of generating RanGTP (33). Interestingly, RanGEF is found mostly in the nucleus (34, 35), although it might be associated with the NPC (Floer, M. and Blobel, G., unpublished observation). Another regulator of Ran is the above mentioned RanGTPase activating protein

RanGAP. RanGAP was originally identified in *S. cerevisiae* by Hartwell (36), and has been termed Rna1 in yeast. A *Rna1* mutant yeast strain was later found to be deficient in RNA export from the nucleus (37). The human homolog, RanGAP1, was later identified and shown to stimulate GTP hydrolysis on Ran more than 1000-fold (28). Interestingly, RanGAP is found mostly in the cytoplasm (28, 38). However, RanGAP has also been found at the nuclear periphery (38, 39). In particular, in mammalian cells RanGAP is modified in an ubiquitin-like manner, which localizes it to Nup358, a nucleoporin that is part of the cytoplasmic fibrils emanating from the NPC (40, 41). The differential localization of RanGEF in the nucleoplasm and RanGAP mainly in the cytoplasm is thought to generate a gradient of RanGTP across the NPC, with RanGTP in the nucleus and RanGDP in the cytoplasm. This RanGTP gradient has been proposed to be one of the determinants for directionality in nuclear transport (42).

1.8 p10/NTF2

The other factor that copurified with fraction B was isolated and termed p10 (43) or NTF2 (44). p10/NTF2 shared homology with a previously identified human protein of unknown function called pp15 (placental protein 15)(45).

p10/NTF2 and Ran together yielded the same translocation activity of a cNLS-protein across the NPC as fraction B (43). p10/NTF2 from fraction B coeluted with Ran on a gel filtration column with an apparent mobility of a 50-60,000 mol-wt protein (43). This finding suggested that cytosolic p10/NTF2 might be in complex with Ran. p10/NTF2 was later found to bind to Ran, when it is in its GDP bound form (46-48) and to interact with certain peptide repeat containing nucleoporins (44, 46, 49). p10/NTF2 has also been shown to be a dimer, and its atomic structure has been solved (50). p10/NTF2 is an essential protein in *S. cerevisiae* (46, 51). However, cells that lack p10/NTF2 are viable if Ran is overexpressed (52). p10/NTF2 has been suggested to function as an adapter for RanGDP recruiting RanGDP to the NPC. Recently, it has also been suggested that p10/NTF2 might function to import RanGDP into the nucleus (53). However, the exact function of p10/NTF2 in nuclear protein import remains elusive.

1.9 Karyopherin α/β1

The active components in fraction A were identified by several investigators. Two factors were found which together mediate docking of a cNLS-protein at the NPC, and were termed karyopherin (54) or nuclear pore

targeting complex (55). The receptor that interacts directly with the cNLS was shown to be a 60,000 mol-wt protein and has been termed karyopherin α (56), NLS-receptor (57) or importin (55, 58). The other factor is a 97,000 mol-wt protein and has been termed karyopherin β (54), p97 (57, 59) or importin β (60, 61). Karyopherin α and β can be isolated as a complex from cytosol (54, 60, 62). Karyopherin β was further shown to interact directly with several peptide repeat containing nucleoporins (63-67). A heterodimeric karyopherin α/β complex has also been isolated from *S. cerevisiae* (68). In yeast the homolog of karyopherin α is called Kap60; the karyopherin β homolog is called Kap95. Interestingly, recombinant karyopherin α and β subunits from *S. cerevisiae* dock a cNLS-containing protein at the nuclear rim of digitonin-permeabilized mammalian cells (68). However, translocation of the docked cNLS-protein cannot be achieved by adding fraction B from mammalian cells indicating that the factors from different species are not fully compatible. During the last years a number of proteins that share a high degree of sequence similarity with karyopherin β have been identified (for review see (69, 70)). Each of these karyopherin β homologs mediates nuclear import or export of distinct proteins, RNA and ribonucleoproteins. The existence of multiple import pathways has interesting implications for our understanding of

the mechanism of nuclear protein import. However, we will not discuss these different pathways further. In chapter 4, however, we will describe nuclear export of proteins, which is mediated by a distinct karyopherin β homolog, the protein Crm1. Since several karyopherin β homologs are known to date, the karyopherin β involved in import of cNLS-containing proteins has been termed karyopherin β1.

1.10 Regulators of Ran are required for nuclear protein import *in vivo*

The fractionation experiments described above led to the identification of four factors that are required for import of a cNLS-protein into the nucleus of digitonin-permeabilized cells. Karyopherin α and β1 mediate docking of the cNLS-protein at the NPC. Subsequent translocation across the NPC occurs in the presence of Ran, p10/NTF2 and GTP. However, it is now known that in the digitonin-permeabilized cell assay used for the fractionation experiments other proteins involved in import were still associated with the NE and were therefore not limiting in the reaction. These proteins were later found to be required for import of a cNLS-containing protein *in vivo*. RanGAP was found to be required for import of a cNLS-protein in living cells, since a mutation in the *RNA1* gene in

S. cerevisiae resulted in defects in nuclear import of a reporter protein (71). Moreover, import of a cNLS-protein into the nucleus of digitonin-permeabilized cells became dependent upon the presence of RanGAP, if Ran had been loaded with GTP prior to its addition to the import reaction (72). This result suggested that RanGTP on the cytoplasmic side of the NPC prevents import of a cNLS-protein, and therefore one function of RanGAP might be to keep cytoplasmic levels of RanGTP low. Interestingly, Mahajan et al. showed that NPC-associated RanGAP is required for import of a cNLS-protein, since import was abolished, when NPC-associated RanGAP was inhibited with an antibody (41). Inhibition of import could not be overcome by adding RanGAP to the cytosolic side of the NPC, indicating that the NPC-associated RanGAP might play a role in nuclear import that is distinct from keeping cytosolic Ran in the GDP bound form. However, it cannot be excluded that the inhibition seen in this experiment was due to occlusion of the NPC by the antibody against RanGAP.

Another factor that is required for import of a cNLS-protein into the nucleus in living cells is the exchange factor RanGEF. Tachibana et al. showed that a cell line termed tsBN2, which carries a temperature sensitive mutant RanGEF protein, shows defects in import of a cNLS-conjugate at the non-

permissive temperature (73). A third factor that has been shown to play a role in nuclear import is the Ran interacting protein RanBP1. RanBP1 was originally identified as a RanGTP binding protein (74). RanBP1 was later shown to stimulate RanGAP induced GTP hydrolysis on Ran 10-fold and to inhibit GTP exchange by RanGEF (75). RanBP1 was found to be required for nuclear import of a cNLS-protein, since cells expressing a mutant RanBP1 protein in *S. cerevisiae* were defective in import of a cNLS-protein (76).

1.11 Interactions between different import factors *in vitro*

First insight into the mechanism of nuclear import of a cNLS-containing protein on a molecular level was provided by *in vitro* studies by Rexach and Blobel (77). Using a GST-solution binding assay with recombinant proteins from *S. cerevisiae* these investigators could detect several complexes between different transport factors that are likely to represent reaction intermediates of nuclear protein import *in vivo*. First, a GST-fusion protein with the cNLS from SV-40 large T-antigen bound to karyopherin α as expected. Interestingly, this interaction was stimulated by karyopherin β1, indicating that karyopherin α/β1 complex has a higher affinity for a cNLS than karyopherin α alone. A karyopherin α/β1

complex was further found to bind to fragments of the nucleoporins Nup1 and Nup2 containing their FXFG-peptide repeat regions. However, binding of karyopherin α/β1 to fragments of Nup145 or Nup57 containing their GLFG-peptide repeat regions could not be detected under these conditions. Interestingly, binding of a cNLS/karyopherin α/β1 complex to the peptide repeat region of Nup1 was accompanied by release of the cNLS-protein. Rexach and Blobel could further show that RanGTP but not RanGDP releases the karyopherin α/β1 complex from the peptide repeat region of Nup1 or Nup2, respectively. Release was accompanied by dissociation of the karyopherin α/β1 complex and formation of a RanGTP/karyopherin β1 complex. Release by RanGTP did not require GTP hydrolysis on Ran, since Ran that had been loaded with the non-hydrolyzable analog GMP-PNP could function in the release reaction as well. It was also found that RanGTP could release the cNLS-protein from the cNLS/karyopherin α/β1 complex. These results suggested that translocation of a docked cNLS-protein across the NPC might involve release of the docked karyopherin α/β1 complex from a nucleoporin via RanGTP *in vivo*. A cNLS/karyopherin α/β1 complex was found to interact with several nucleoporins, many of which are glycosylated (78-80). Considering the inhibition of

translocation of a docked cNLS-protein across the NPC in the presence of WGA (16), this result might suggest that a cNLS-protein containing complex interacts with these repeat containing nucleoporins during translocation. These findings led to the hypothesis that translocation across the NPC might occur via several cycles of docking and release by RanGTP until the cNLS-protein reaches the nuclear side of the NPC. This model invokes a translocation mechanism by guided diffusion along the cytonucleoplasmic axis of the NPC. However, it has also been suggested that RanGTP releases karyopherin α/β1 from the NPC and dissociates the cNLS/karyopherin α/β1 complex only on the nucleoplasmic side of the NPC (72). Further studies are necessary to elucidate this question. Another question that awaits further clarification concerns the observation that the cNLS-protein is released when cNLS/karyopherin α/β1 interacts with certain nucleoporins *in vitro* (77). It is not clear whether a cNLS-protein is also released upon docking of a cNLS/karyopherin α/β1 complex at the NPC *in vivo*. However, one might speculate that release of the cNLS-protein upon docking would allow its diffusion to a next docking site independently of the release of karyopherin α/β1 by RanGTP accelerating its translocation across the NPC.

While these studies by Rexach and Blobel were in progress we started to

analyze GTP hydrolysis and nucleotide exchange on Ran and the effect of different transport factors on these reactions. GTP hydrolysis and exchange on Ran were likely to be affected by the interaction of RanGTP with other factors, in analogy to what was known for other GTP binding proteins. We investigated the effect of other transport factors on GTP hydrolysis and exchange on Ran to further our understanding of the mechanism of nuclear protein import and export.

Chapter 2: Experimental procedures

2.1 Protein expression and purification

The gene encoding for yeast Ran, *GSP1*, (81) was amplified from *S. cerevisiae* genomic DNA (Promega) using Taq polymerase (Boehringer Mannheim) and the primers 5'-ATA TCC ATG GCT TCT GCC CCA GCT GCT AAC-3' and 5'-GTT GGA TCC TTA TAA ATC AGC ATC ATC-3'. The PCR product was digested with *Nco*I and *Bam*HI and inserted into *Nco*I/*Bam*HI-digested pET21d vector (Novagen). The plasmid was introduced into *E. coli* strain BLR(DE3)(Novagen). Recombinant Ran was purified as follows. Cells were grown in 2 liters of LB medium containing 200 μg/ml of ampicillin at 37°C to a cell density of 0.6 A_{600} units. Isopropyl-1-thio-β-D-galactopyranoside (IPTG) was added to 0.1 mM to induce expression of the protein. After 3 h cells were harvested by centrifugation at 2,000 x *g* at 4°C, and the cell pellet was resuspended in 25 ml of ice cold Tris-buffer containing 10 mM Tris-HCl, pH 8.0, 1 mM $MgCl_2$ and 1 mM DTT. Aprotinin, pepstatin and leupeptin were added to 1 μg/ml and PMSF to 0.1 mM. All subsequent operations were carried out at 4°C. Cells were lyzed in a French pressure cell at 9,000 psi and the lysate was centrifuged for 20 min at 10,000 x *g*. Ran was precipitated from the supernatant fraction at 25-55 %

ammonium sulfate saturation and centrifuged at 10,000 x *g* for 10 min. The pellet was resuspended in 25 ml of Tris-buffer and dialyzed overnight against Tris-buffer. To load that fraction of Ran that might be free of nucleotide, the dialyzed fraction was incubated in the presence of 0.5 mM GTP and 10 mM $MgCl_2$ for 10 min at 4°C. After centrifugation at 10,000 x *g* the cleared supernatant fraction was subjected to chromatography on a MonoQ FPLC column (AmershamPharmacia) at a flow rate of 1 ml/min using a linear gradient from 0-500 mM NaCl in Tris-buffer. Ran eluted between 230-280 mM NaCl as assayed by SDS-PAGE. The fractions containing Ran were pooled (~ 2 ml) and concentrated in a Centricon 10 concentrator (Amicon) to 100 µl. This solution was sieved on a Superdex75 FPLC column (HR 30/10; AmershamPharmacia) equilibrated with buffer A (20 mM Hepes, pH 7.3, 110 mM KOAc, 2 mM $Mg(OAc)_2$, 1 mM EGTA and 2 mM DTT). Peak fractions containing 1-2 mg/ml of Ran were pooled (~ 2 ml), and 20 µl aliquots were frozen in liquid nitrogen and stored at -80°C.

The RanGAP expression vector was kindly provided by Ulf Nehrbass and had been constructed in the following manner. The *RNA1* gene was amplified by PCR from yeast genomic DNA (Promega) using synthetic oligonucleotides 5'-TTA GGA TCC GCT ACC TTG CAC TTC GTT-3' and 5'-CCA GAA TTC ATT

GTG CTA CTT GGA GCC-3' introducing a *Bam*HI-site in frame with the initiation codon and an *Eco*RI site after the stop codon. The *Bam*HI/*Eco*RI fragment was ligated into vector pGEX-2TK (AmershamPharmacia) and thereby fused with the glutathione S-transferase gene (GST). The plasmid was introduced into *E. coli* strain BLR(DE3)(Novagen). For purification of the recombinant RanGAP cells were grown in 2 liters of LB medium containing 200 µg/ml of ampicillin at 30°C to a cell density of 0.5 A_{600} units. IPTG was added to 0.2 mM and cells were grown for 3 h. Cells were harvested by centrifugation at 2,000 x *g* at 4°C, and the cell pellet was resuspended in 25 ml of PBS (140 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4, pH 7.3, 1 mM DTT). Cells were lyzed using a French pressure cell, and the lysate centrifuged at 10,000 x *g* for 10 min. The supernatant fraction was applied to a 1 ml glutathione-Sepharose column (AmershamPharmacia) equilibrated in PBS at 4°C. After washing with 100 ml PBS the GST-fusion protein was cleaved on the column by incubation at 21°C for 12 hr with 10 NIH units of thrombin (Sigma). The eluate of 1.5 ml contained 0.9 mg/ml protein, and 50 µl aliquots were frozen in liquid nitrogen and stored at -80°C.

Yeast karyopherin α (Kap60) and karyopherin β1 (Kap95), the Nup1

fragment containing a FXFG repeat region (AA 432-816) and the Nup2 fragment containing its FXFG repeat region (AA 186-561), but lacking its RBH domain, were expressed as GST-fusion proteins as described (68, 77). The expression vector containing a GST-fusion with full length Nup36 was kindly provided by Ulf Nehrbass (46). Proteins were purified and the GST-moiety was cleaved with thrombin (Sigma) as described for GST-fusion proteins (77, 82).

Nsp1 was expressed as a GST-fusion protein. The gene encoding Nsp1 was amplified by PCR using primers 5'T TTA GGA TCC ATG AAC TTC AAT ACA CC3' and 5'G TCC CCC GGG TCA TTT CTT TAT CGA GT3' from *S. cerevisiae* genomic DNA (Promega). The resulting PCR product was inserted into pGEX-4T3 (AmershamPharmacia) as a *Bam*HI/*Sma*I fragment. The protein was expressed in *E. coli* strain BL21(DE3)(Novagen), purified on 10 ml glutathione-Sepharose beads (Amersham/Pharmacia) and eluted with 10 mM glutathione as described for GST-fusion proteins (68). The 10 ml eluate contained 0.8 mg/ml protein and was dialyzed against buffer A. The purified protein was stored in frozen aliquots at -80°C.

The expression vector containing full length Nup2 was kindly provided by Michael Rexach and had been constructed in the following manner. The gene

encoding Nup2 was amplified by PCR from *S. cerevisiae* genomic DNA (Promega) using primers 5'CCG GGA TCC ATG GCC AAA AGA GTT GCC GAT GCG CAA ATA3' and 5'CCG GAA TTC TTA TTT CAT TTC TTT TTT AGC ATC TTC AAT AG3'. The PCR fragment was inserted as a *Bam*HI/*Eco*RI fragment into pGEX-2TK (AmershamPharmacia). The resulting GST-fusion protein was expressed in *E. coli* strain BL21(DE3)(Novagen), purified on 15 ml glutathione-Sepharose beads (Amersham/Pharmacia) and eluted with 10 mM glutathione as described for GST-fusion proteins (68). The 20 ml eluate contained 0.2 mg/ml and was dialyzed against buffer A. The purified protein was stored in frozen aliquots at –80°C.

The C-terminus of Nup1 (C-Nup1) was expressed as a GST-fusion protein. The fragment of the *NUP1* gene encoding amino acids 963-1076 was amplified by PCR using primers 5'CCG GGA TCC CCA CAT CAA TCG CAA ACC CCA TCT TTC3' and 5'CCG GAA TTC TTA CCT TTT AGA GTG CCT CAT TCT TGC AAT3' from *S. cerevisiae* genomic DNA (Promega). The resulting PCR fragment was inserted into pGEX-2TK (AmershamPharmacia) as a *Bam*HI/*Eco*RI fragment. The GST-fusion protein of C-Nup1 was purified on 10 ml glutathione-Sepharose beads (AmershamPharmacia) and eluted with 10 mM glutathione, as

previously described for GST-fusion proteins (68). The 10 ml eluate contained 0.2 mg/ml protein and was dialyzed against buffer A. The purified protein was stored in frozen aliquots at -80°C.

RanGEF (yeast Prp20) was expressed as a GST-fusion protein. The gene encoding this protein was amplified by PCR using primers 5'GGA AGA TCT ATG GTC AAA AGA ACA GTC GCC ACC AAT G3' and 5'CCG GAA TTC TTA ATC ATC CAT TTC ATC CGC TCT CTT TTC3' from *S. cerevisiae* genomic DNA (Promega). The RanGEF PCR product was inserted into vector pGEX-2TK (AmershamPharmacia) as a *Bgl*II/*Eco*RI fragment. The protein was expressed in *E. coli* strain BLR (Novagen) and purified from bacterial lysate on glutathione-Sepharose beads (AmershamPharmacia). The GST moiety was cleaved with thrombin (Sigma) as described for GST-fusion proteins in (77, 82). The cleaved protein was eluted from the beads with buffer A. Then the thrombin inhibitor hirudin (Sigma) was added to 10 ml eluate containing 0.1 mg/ml of protein. The purified protein was stored in frozen aliquots at -80°C.

Yeast RanBP1 (Yrb1) was amplified from *S. cerevisiae* genomic DNA (Promega) by PCR and inserted as a *Nco*I/*Bam*HI fragment into pET-21d vector (Novagen). Protein was expressed in *E. coli* strain BLR(DE3)(Novagen) at 37°C

for 4 h. Cells were harvested by centrifugation at 2,000 x *g* and the cell pellet was resuspended in ice cold Tris buffer (10 mM Tris-HCl, pH 6.8, 1 mM $MgCl_2$ and 1 mM DTT). After cell lysis using a French pressure cell ammonium sulfate was added at a final concentration of 55 %; RanBP1 was found in the soluble fraction. The dialyzed 10,000 x *g* supernatant was loaded onto a MonoQ FPLC column (AmershamPharmacia) and proteins were eluted using a linear gradient (0-500 mM) of NaCl in Tris buffer. RanBP1 eluted between 50 and 200 mM NaCl. Fractions containing RanBP1 were pooled, concentrated with a centricon 10 unit (Amicon), and fractionated on a Superdex75 FPLC column (Amersham Pharmacia) which was equilibrated with buffer A containing 150 mM KOAc. RanBP1 eluted as a dimer with a mobility equal to that of a 70,000 mol-wt globular protein. Fractions containing 2 mg/ml of RanBP1 (~ 15 ml) were pooled and aliquots were stored at -80°C.

Crm1 was expressed as a GST-fusion protein. The gene encoding Crm1 was amplified by PCR from *S. cerevisiae* genomic DNA (Promega) using primers 5'ATA GGA TAA CAT GGA TCC GAA GGA ATT TTG GAT TTT3' and 5'AAA AAT ATT GGA AAT TTA AAG AAT GAT ACG CCA GTC GAC CTA ATC ATC AAG TTC GGA3'. The PCR product was inserted as a *Bam*HI/*Sal*I

fragment into vector pGEX-4T3 (Amersham/Pharmacia), into which a Tev protease cleavage site had been inserted (this vector was kindly provided by Y.M. Chook and will be described elsewhere). The protein was expressed in *E. coli* strain DH5α (Novagen). The GST-fusion protein was purified on 10 ml glutathione-Sepharose beads (Amersham/Pharmacia), and the GST moiety was cleaved by incubation with 1,000 units of Tev protease (GibcoBRL) per 20 mg of protein for 12 h at 21°C. The cleaved protein was eluted from the beads with buffer A. This resulted in 10 ml eluate containing 0.9 mg/ml protein. The purified protein was stored in frozen aliquots at –80°C.

To obtain recombinant Nup42 protein the gene encoding Nup42 was amplified from *S. cerevisiae* genomic DNA (Promega) by PCR using primers 5'A GGT GGA TCC TCA GCT TTC GGT AAC CCA3' and 5'TCC GCG GCC GCT ATG GCC GAC GTC GAC CTA TGC AAC CAA TGC AGG3'. The resulting PCR product was inserted into pGEX-4T3 (AmershamPharmacia) as a *Bam*HI/*Sal*I fragment. The protein was expressed in *E. coli* strain DH5α (Novagen), and purified as described for other GST-proteins (82). After cleavage with thrombin (Sigma) the protein was eluted from the beads with buffer A. This resulted in 10 ml eluate containing 0.15 mg/ml protein. After addition of hirudin

(Sigma) the protein was stored in frozen aliquots at –80°C.

Recombinant Nup116 was expressed as a GST-fusion protein. The gene encoding Nup116 was amplified from *S. cerevisiae* genomic DNA (Promega) by PCR using primers 5'GGA TCC AGA TCT TTT GGA GTT AGC CGT GGC3' and 5'TAA AGA CTC GAG TCA GGT CTG CTC TGC AGC3'. The PCR product was inserted as a *Bgl*II/*Xho*I fragment into vector pGEX-4T3 (AmershamPharmacia). The GST-fusion proteins was expressed in *E. coli* strain BL21(DE3)(Novagen). The protein was purified on 10 ml glutathione-Sepharose beads (AmershamPharmacia) and eluted with 10 mM glutathione as described for other GST-fusion proteins (68). This resulted in 4 ml eluate containing 0.25 mg/ml protein. The purified protein was stored in frozen aliquots at -80°C.

The fragment of Nup159 containing its FG-peptide repeat region (AA 441-876) was expressed as a His-fusion protein as described (65). The protein was purified on a His-Trap column (AmershamPharmacia) using a linear gradient (5-500 mM) imidazole in buffer A that did not contain DTT. The protein eluted between 200 and 250 mM imidazole. Fractions containing Nup159 were pooled, concentrated in an Ultrafree 15 concentrator (Millipore), and fractionated on a Superdex200 fast liquid chromatography column (AmershamPharmacia), which

was equilibrated with buffer A containing 1 mM DTT. Fractions (~ 5 ml) containing 1 mg/ml Nup159 were pooled and frozen aliquots were stored at -80°C.

To obtain GST-Ran the gene encoding Ran was amplified by PCR using primers 5'TTA GGA TCC TCT GCC CCA GCT GCT AAC3' and 5'C GTT GGA TCC TTA TAA ATC AGC ATC ATC TT3'. The PCR product was inserted as a *Bam*HI fragment into vector pGEX-2TK (AmershamPharmacia). The protein was purified as described for GST-fusion proteins (82) in PBS buffer (140 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4 [pH 7.3]). This resulted in 5 ml eluate containing 1 mg/ml protein. The purified protein was stored in frozen aliquots at -80°C. GST-Ran was found to be mostly nucleotide free in PBS buffer, as determined by a method described below. GST-Ran could be reloaded with GTP by incubation with 1 mM GTP in the presence of 5 mM $Mg(OAc)_2$ for 1 h at 21°C. Free nucleotide was removed by dialysis against buffer A containing 1 mM DTT. This resulted in GST-Ran that was mostly GTP bound (80-90 %).

HIV-1 Rev protein was purchased from Intracel.

2.2 Determination of GTP/GDP bound to recombinant Ran

Aliquots of frozen Ran solution were applied to a Beckman System Gold

and a C18 reversed-phase column (4.6 mm x 15 cm; Rainin) equilibrated with triethylamine-buffer (100 mM triethylamine, pH 6.5, phosphoric acid). Using a gradient from 0-20 % acetonitrile in triethylamine-buffer at a flow rate of 1 ml/min GDP eluted at 8.6 min and GTP at 9.4 min. Quantitation was by measuring A_{254}.

2.3 GTP hydrolysis and nucleotide exchange assays

5 μM Ran were incubated for 30 min on ice with 30 nM [γ-^{32}P]GTP (6000 Ci/mmol, NEN) in buffer B containing 5 mM EDTA, 20 mM Hepes pH 7.3, 100 mM KOAc and 1 mM DTT in a final volume of 100 μl followed by the addition of 20 mM $MgCl_2$. Unbound nucleotide was removed by gel filtration on a G50 Sephadex (AmershamPharmacia) column prepared in a 1 ml syringe equilibrated with buffer C (20 mM Hepes, pH 7.3, 100 mM KOAc, 20 mM $Mg(OAc)_2$, 1 mM DTT and 0.5 mg/ml BSA). This yielded ~ 100 μl of 1.5 μM Ran solution containing 200 nM Ran[γ-^{32}P]GTP as determined on the reversed-phase column.

No preincubation: Ran-[γ-^{32}P]GTP was incubated either in the absence or presence of various amounts of RanGAP and karyopherin β1 as indicated in the Figure Legends in buffer C in a final volume of 100 μl at 21°C for 10 min. With

preincubation: Ran-[γ-^{32}P]GTP and karyopherin β1, Crm1 or nups, as indicated in the Figure Legends were preincubated for 10 or 15 min at 21°C, and then RanGAP, karyopherin α, C-Nup1 or RanBP1 were added as indicated in the Figure Legends in a final volume of 100 µl. Reactions were incubated for 20 min at 21°C. To determine the dissociation constants for RanGTP complexes we also measured inhibition of GTP hydrolysis after 2 min reaction time. However, this did not have an effect on the K_D values. After incubation 20 µl aliquots were diluted into 1 ml of ice cold buffer C and filtered through nitrocellulose (Schleicher and Schuell). Filters were washed with 5 ml buffer C, dried and radioactivity was counted in the presence of 3 ml scintillation fluid (Ready Safe, Beckman) on a Wallac 1410 scintillation counter.

Nucleotide exchange assays were performed essentially as the GTP hydrolysis assays, except that Ran was labeled with [α-^{32}P]GTP. Ran-[α-^{32}P]GTP was incubated with karyopherin β1, Crm1, RanBP1 or nups as indicated in the Figure Legends for 10 or 15 min at 21°C, and then RanGEF, karyopherin α, C-Nup1, or RanBP1 were added together with 200 µM GTP and 200 µM GDP. Reactions were incubated for 20 min at 21°C. 20 µl aliquots were diluted, filtered and the radioactivity was counted as described for hydrolysis assays.

2.4 Determination of complex formation between karyopherin β1 and RanGTP or RanGDP

For these experiments the nucleotide exchange reaction for Ran was done in the presence of a 10 fold molar excess of either GDP or GTP over Ran. To allow for complex formation 2.5 µM karyopherin β1 and either 5 µM RanGTP or RanGDP were incubated in a final volume of 200 µl of buffer A for 10 min at 21°C. The reaction mixture was then subjected to gel filtration on a Superdex200 column (HR30/10; AmershamPharmacia) equilibrated with buffer A. Fractions of 0.5 ml were collected, precipitated with 10 % trichloroacetic acid (TCA) and subjected to SDS-PAGE followed by staining of the gel with Coomassie Blue. In a control experiment Ran was first labeled with either [8,5'-^{3}H]GDP or [γ-^{32}P]GTP. For this 10 µM Ran was incubated in the presence of either 10 nM [8,5'-^{3}H]GDP (34 Ci/mmol, NEN) or 10 nM [γ-^{32}P]GTP (6000 Ci/mmol, NEN) in buffer B for 30 min at 21°C. After addition of 20 mM $Mg(OAc)_2$, 5 µM Ran[8,5'-^{3}H]GDP or 5 µM Ran[γ-^{32}P]GTP was incubated with 2.5 µM karyopherin β1 for 10 min at 21°C. The reaction mixture was subjected to gel filtration as described and the elution of radioactivity was monitored by measuring 10 µl aliquots of each fraction in a scintillation counter.

2.5 Solution binding assays

A, GST-C-Nup1 immobilization: For each experiment, an *E. coli* lysate containing GST-C-Nup1 was incubated for 20 min at 4°C with glutathione-agarose beads (Sigma)(2 µg of GST-C-Nup1 per 10 µl of beads) in 0.5 ml of binding buffer 1 (20 mM Hepes, pH 6.8, 150 mM KOAc, 2 mM $Mg(OAc)_2$, 1 mM DTT, 0.1 % Tween 20, 0.1 % casaminoacids). Beads were washed 6 times by centrifugation (2,000 x *g* for 30 sec) and resuspension in 0.5 ml of chilled binding buffer 1. One-step assay: 20 µl of bead slurry was added to siliconized 0.5 ml tubes (Sigma) that contained protein additions as indicated in the Figure Legends, in a total volume of 40 µl. Tubes were tumbled end over end for 1 h at 4°C or 21°C as indicated in the Figure Legends. Two-step assay: the bead slurry was incubated for 15 min at 4°C with karyopherin β1 (0.6 µg per 10 µl of beads) in 1 ml of binding buffer 1. After washing 3 times with 0.5 ml of binding buffer 1, beads were resuspended as a 50 % slurry and incubated with protein additions as in the one-step assay. Beads were subjected to centrifugation at 2,000 x *g* for 30 sec, and unbound proteins were collected by removing 30 µl from the meniscus: this constitutes the unbound fraction. Beads were washed twice with 0.5 ml of chilled binding buffer 1 as before, and 28 µl of binding buffer 1 was added; this

constitutes the bound fraction. 12 µl or 10 µl of 6 x sample loading buffer (60 % glycerol, 30 % SDS, 0.0075 % bromophenol blue, 6 M Tris-HCl pH 6.8, 1 M 2-mercaptoethanol) was added to the bound and unbound fractions, respectively. After incubation at 95°C for 15 min, proteins in 18 µl of each sample were resolved by SDS-PAGE and stained with Coomassie blue. *B,* GST-Ran immobilization: GST-RanGTP was incubated with glutathione-agarose beads (Sigma) for 20 min at 21°C (7 µg GST-RanGTP per 10 µl of beads) in 0.5 ml of binding buffer 2 (150 mM KOAc, 20 mM Hepes [pH 7.3], 2 mM $Mg(OAc)_2$, 1 mM DTT, 0.1 % Tween 20 and 0.1 % casaminoacids). One step assay: After washing with binding buffer 2, 10 µl beads per reaction was incubated with other proteins as indicated in the Figure Legends for 30 min at 21°C. Two step assay: After washing with binding buffer 2, the immobilized GST-Ran was incubated with 1.7 µM RanGEF for 20 min at 21°C. The beads were washed and 10 µl of beads per reaction was incubated with GTP and other proteins as indicated in the Figure Legends for 10 min at 21°C. The unbound fraction was collected by removing 28 µl from the meniscus after centrifugation at 2,000 x *g* for 1 min. The beads were washed twice with binding buffer 2. Then 18 µl of binding buffer 2 was added; this constitutes the bound fraction. 6 µl of 6 x sample loading buffer

was added to the bound and unbound fractions. After incubation at 95°C for 10 min 14 µl of each sample was resolved by SDS-PAGE using 4-20 % gradient gels and stained with Coomassie Blue.

2.6 Preparation of RanGTP and RanGDP for solution binding assays

6 µM Ran was incubated in the presence of 600 µM GTP or GDP in buffer B containing 15 mM EDTA for 90 min at 21°C. The reaction was stopped by adding 30 mM $Mg(OAc)_2$ and the samples were incubated for 15 min at 4°C. Frozen aliquots were stored at –80°C.

2.7 Surface plasmon resonance experiments

The surface plasmon resonance methodology is described elsewhere (83). Experiments were conducted on a BIAcore (upgrade) instrument (BIAcore Inc.). Ran was immobilized on a CM5 sensor chip (BIAcore Inc.; research grade) by amine coupling. The surface was activated with 5 µl of a 1:1 mixture of 0.05 M N-hydrosuccinimide and 0.2 M N-ethyl-N'-(3-dimethylamino-propyl)-carbodiimide hydrochloride at a flow rate of 5 µl/min. Ran was coupled by injecting 30 µl of a solution containing 10 µg/ml Ran in 10 mM NaOAc, pH 5.0, 2

mM $Mg(OAc)_2$. The surface was blocked by injecting 30 µl of 300 mM Tris-HCl pH 8.0, 0.1 mM GTP, 2 mM $Mg(OAc)_2$. Typically, this resulted in immobilization of 300-400 resonance units (RU) of Ran on the sensor chip surface. We determined that only ~ 40 % of the Ran was GTP bound and ~ 60 % was GDP bound; therefore we estimate that 120-160 RU of RanGTP were immobilized. All experiments were performed in buffer A containing 1 mM EGTA, 0.005 % Tween 20 and 0.1 mM GTP at a flow rate of 10 µl/min at 25°C. The Ran surface was regenerated by injecting 5 µl of 0.5 % Triton X-100 and 0.01% SDS in water. To obtain RanGMP-PCP, 30 µM Ran was incubated for 1 h at 21°C in 0.5 ml buffer A with 5 mM EDTA, 2 mM GMP-PCP and no $Mg(OAc)_2$. The exchange reaction was quenched by adding 20 mM $MgCl_2$, and unbound nucleotide was removed on a Nap5 spin column (Pharmacia). This resulted in Ran containing ~ 39 % GMP-PCP, ~ 60 % GDP and less than 1 % GTP. A RanGMP-PCP surface was generated as described for Ran.

2.8 Determination of protein concentrations

Protein concentrations were determined by the method of Bradford (84) using bovine serum albumin as a standard.

Chapter 3: Mechanism of nuclear protein import – recycling of import factors

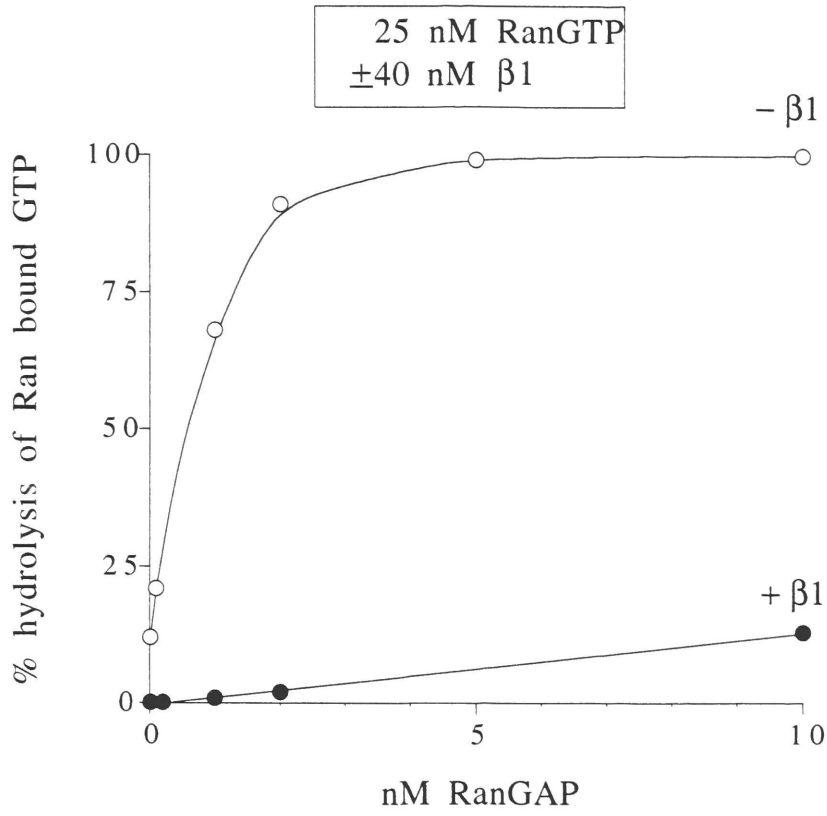
3.1 Results

3.1.1 Karyopherin β1 inhibits RanGAP stimulated GTP hydrolysis on Ran by forming a complex with RanGTP

Ran, RanGAP and karyopherin β1, all from *S. cerevisiae*, were expressed as recombinant proteins in *E. coli* (see Experimental Procedures). Each of the purified recombinant proteins yielded a single band of the expected electrophoretic mobility upon SDS-PAGE analysis (not shown). The purified recombinant Ran was determined to contain 40 % GDP and 25 % GTP whereas 35 % is nucleotide free (not shown). To assay for GTP hydrolysis, the endogenous Ran bound GDP or GTP were in part exchanged with [γ-^{32}P]GTP (see Experimental Procedures). After incubation with RanGAP, GTP hydrolysis was measured using a nitrocellulose filter binding assay in which hydrolyzed ^{32}Pi is not retained by the filter whereas Ran bound, non hydrolyzed [γ-^{32}P]GTP is retained. Incubation of 25 nM Ran-[γ-^{32}P]GTP with 1 nM RanGAP yielded 70 % GTP hydrolysis and 2 nM RanGAP resulted in near 100 % GTP hydrolysis within the 10 min reaction time (Fig. 2). Strikingly, in the presence of 40 nM karyopherin β1, the RanGAP

Fig. 2 RanGAP stimulated GTP hydrolysis on Ran is inhibited by karyopherin β1.

GTP hydrolysis on Ran was assayed as described in the Experimental Procedures by incubating 25 nM Ran-[γ-^{32}P]GTP in the presence of increasing amounts of RanGAP with (closed circles) or without (open circles) 40 nM karyopherin β1 for 10 min at 21°C. The extent of GTP hydrolysis was determined as described in the Experimental Procedures.


25 nM RanGTP
±40 nM β1
– β1
+ β1
100
75
50
25
0
0
5
10
% hydrolysis of Ran bound GTP
nM RanGAP

stimulated GTP hydrolysis was completely inhibited (Fig. 2).

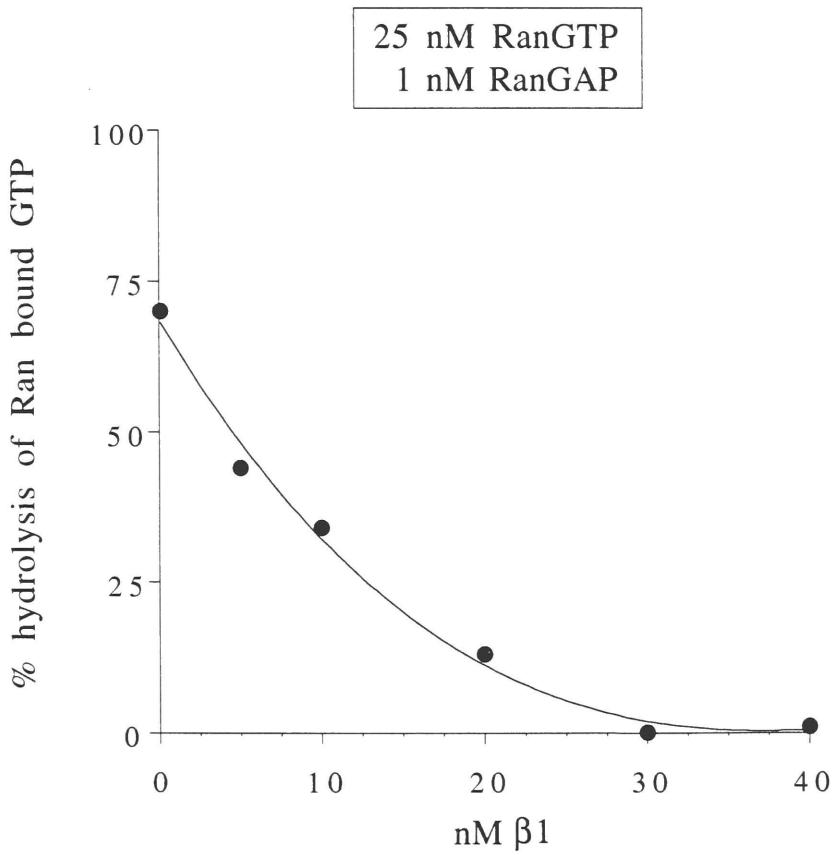
To determine whether karyopherin β1 inhibits RanGAP stimulated GTP hydrolysis in a concentration dependent manner 25 nM Ran-[γ-^{32}P]GTP was incubated with 1 nM RanGAP and increasing amounts of karyopherin β1. Inhibition of RanGAP stimulated GTP hydrolysis was maximal at 30 nM to 40 nM karyopherin β1 (Fig. 3). Increasing the concentration of RanGAP did not overcome the inhibition of karyopherin β1 (Fig. 2). These data suggest that karyopherin β1 did not inhibit RanGAP stimulated GTP hydrolysis by interacting with RanGAP directly, but rather by forming a complex with RanGTP.

3.1.2 Karyopherin β1 forms a high affinity 1:1 complex with RanGTP and a lower affinity complex with RanGDP

To directly test for association of Ran with karyopherin β1 we carried out gel filtration experiments. For these experiments the endogenous Ran bound GDP or GTP was exchanged in the presence of a 10-fold molar excess of either GTP or GDP to assure that most of the Ran would be bound either to GTP or GDP. To allow for complex formation 2.5 μM karyopherin β1 was incubated either with 5 μM RanGTP or RanGDP for 10 min at 21°C. In a control reaction 2.5 μM

Fig. 3 Inhibition of RanGAP stimulated GTP hydrolysis is dependent upon karyopherin β1 concentration.

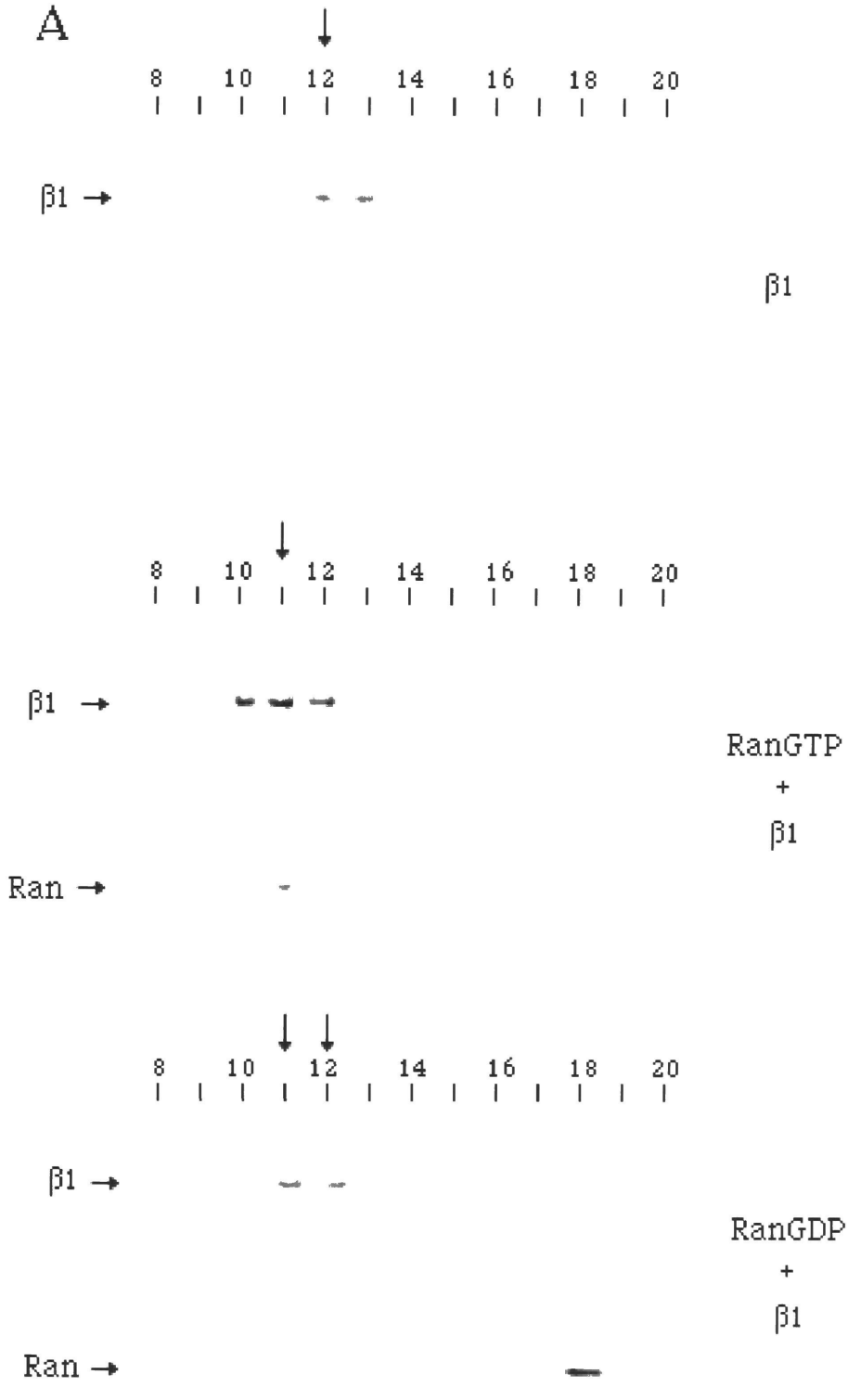
GTP hydrolysis was determined as described in the Experimental Procedures by incubating 25 nM Ran-[γ-^{32}P]GTP in the presence of 1 nM RanGAP and increasing amounts of karyopherin β1 for 10 min at 21°C. The extent of GTP hydrolysis was determined as described in the Experimental Procedures.

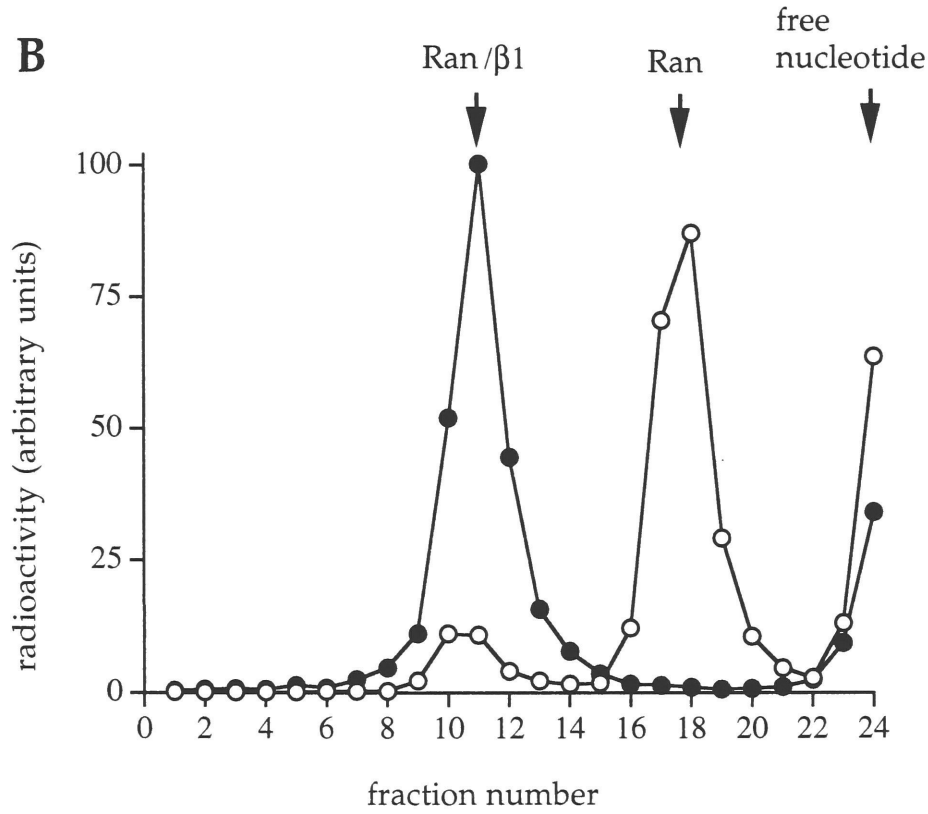

25 nM RanGTP
1 nM RanGAP
% hydrolysis of Ran bound GTP
100
75
50
25
0
0
10
20
30
40
nM β1

karyopherin β1 was incubated without RanGTP or RanGDP. Each of the reaction mixtures was then subjected to gel filtration on a Superdex200 FPLC column and fractions were collected, TCA-precipitated and analyzed by SDS-PAGE and Coomassie blue staining of the gel. The bulk of karyopherin β1 eluted at fraction 12 and 13 (Fig. 4*A*, upper panel). When preincubated with a two-fold molar excess of RanGTP, there was a shift of the karyopherin β1 peak to fractions 10 and 11. Moreover, karyopherin β1 coeluted with about half of the RanGTP, whereas the other half of RanGTP peaked at fraction 18 (Fig. 4*A*, middle panel). These data indicate that karyopherin β1 and RanGTP form a stoichiometric complex. Some binding to karyopherin β1 could also be detected when Ran was loaded with GDP prior to incubation (Fig. 4*A*, lower panel). However, we found that under our exchange conditions there was still about 10 % GTP bound to Ran, as determined on the reversed-phase column. To determine whether there was indeed binding of RanGDP to karyopherin β1, Ran was loaded with [8,5'-^{3}H]GDP prior to incubation with karyopherin β1. Gel filtration on the Superdex200 column resulted in cofractionation of some of the radioactivity with the Ran/karyopherin β1 complex (Fig. 4*B*, open circles). When Ran was loaded with [γ-^{32}P]GTP prior to incubation with karyopherin β1, all the labeled Ran

Fig. 4 Ran forms a complex with karyopherin β1 that can be detected by gel filtration.

Complex formation between Ran and karyopherin β1 was analyzed on a Superdex200 FPLC column as described in the Experimental Procedures. 13 of 24 fractions were analyzed by SDS-PAGE. *A*, Fractions were analyzed by electrophoresis on a 12 % polyacrylamide gel and stained with Coomassie Blue. *Upper panel*,, 2.5 μM karyopherin β1 was incubated in buffer A for 10 min at 21°C. *Middle panel*, 2.5 μM karyopherin β1 was incubated with 5 μM RanGTP for 10 min at 21°C. *Lower panel*, 2.5 μM karyopherin β1 was incubated with 5 μM RanGDP for 10 min at 21°C. *B*, 2.5 μM karyopherin β1 was incubated with 5 μM Ran-[8,5'-^{3}H]GDP (open circles) or 5 μM Ran-[γ-^{32}P]GTP (closed circles) for 10 min at 21°C. Elution of radioactivity was monitored by counting 10 μl aliquots of each of the 24 fractions in a scintillation counter.


A
8
10
12
14
16
18
20
β1 →
β1
RanGTP
+
β1
Ran →
RanGDP
+
β1


B
Ran /β1
Ran
free
nucleotide
100
75
50
25
0
radioactivity (arbitrary units)
0
2
4
6
8
10
12
14
16
18
20
22
24
fraction number

cofractionated with the Ran/karyopherin β1 complex (Fig. 4*B*, closed circles). These results indicate that RanGDP also binds to karyopherin β1, but with much lower affinity than does RanGTP.

3.1.3 Disassembly of the RanGTP/karyopherin β1 complex

We have shown that RanGTP forms a complex with karyopherin β1. Estimates from the inhibition of RanGAP by karyopherin β1 at low concentrations of RanGTP indicate an affinity constant below 1 nM for the RanGTP/karyopherin β1 interaction (Floer, M and Blobel, G., unpublished observation)(0.3 nM for mammalian proteins (72)). How is the RanGTP/karyopherin β1 complex disassembled to allow recycling of both factors for another round of function? We investigated whether disassembly of RanGTP/karyopherin β1 complex is stimulated by other proteins involved in nuclear protein transport. As RanGAP is synthetically lethal with the C-terminus of the nucleoporin Nup1 (85) and with karyopherin β1 (85), we investigated whether the C-terminus of Nup1 and RanGAP are involved in the disassembly of the RanGTP/karyopherin β1 complex.

3.1.4 RanGTP/karyopherin β1 complex is disassembled in the presence of karyopherin α, RanGAP and the C-terminus of Nup1

Karyopherin β1 binds to the C-terminus of Nup1 (C-Nup1)(AA 963-1076)(Fig. 5, lane 1, see also Fig. 12*A*) and is released in the presence of RanGTP (lane 2 and 3) but not RanGDP (lane 4 and 5). This indicates that complex formation of RanGTP with karyopherin β1 abolishes the interaction of karyopherin β1 with C-Nup1. Neither RanGDP nor RanGTP bound to C-Nup1 (not shown). Preincubation of karyopherin β1 with RanGTP for 15 min at 4°C also abolished binding of karyopherin β1 to C-Nup1 (Fig. 6*A*, compare lane 1 to lane 2). We used this observation as an assay to detect disassembly of the RanGTP/karyopherin β1 complex in the presence of different transport factors. Addition of RanGAP to karyopherin β1 and RanGTP that had been preincubated did not stimulate disassembly of the RanGTP/karyopherin β1 complex as judged by the inability of karyopherin β1 to bind to C-Nup1 (lane 3). However, addition of karyopherin α led to binding of some karyopherin β1 to C-Nup1 (lane 4). Karyopherin α bound directly to C-Nup1 in the absence of karyopherin β1 (not shown). Most importantly, when karyopherin α and RanGAP were added together, karyopherin β1 binding to C-Nup1 was restored (lane 5). These results

Fig. 5 Karyopherin β1 binds to the C-terminus of Nup1 (C-Nup1) and binding is abolished by RanGTP but not RanGDP.

Immobilized GST-C-Nup1 (1.25 μM final concentration) was preincubated with 0.15 μM karyopherin β1 for 40 min at 4°C. After washing, the beads were incubated for 40 min at 21°C with no addition (lane 1), 0.6 μM RanGTP (lane 2), 2 μM of RanGTP (lane 3), 0.6 μM RanGDP (lane 4) or 2 μM RanGDP (lane 5). RanGDP and RanGTP were prepared as described in the Experimental Procedures. Bound and unbound fractions were analyzed by SDS-PAGE and Coomassie blue staining.

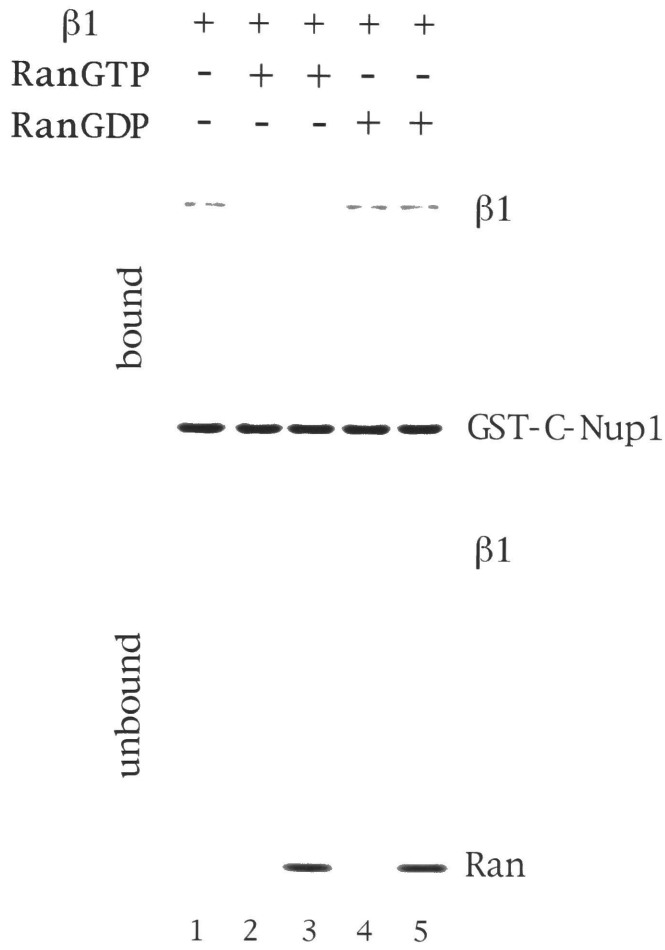
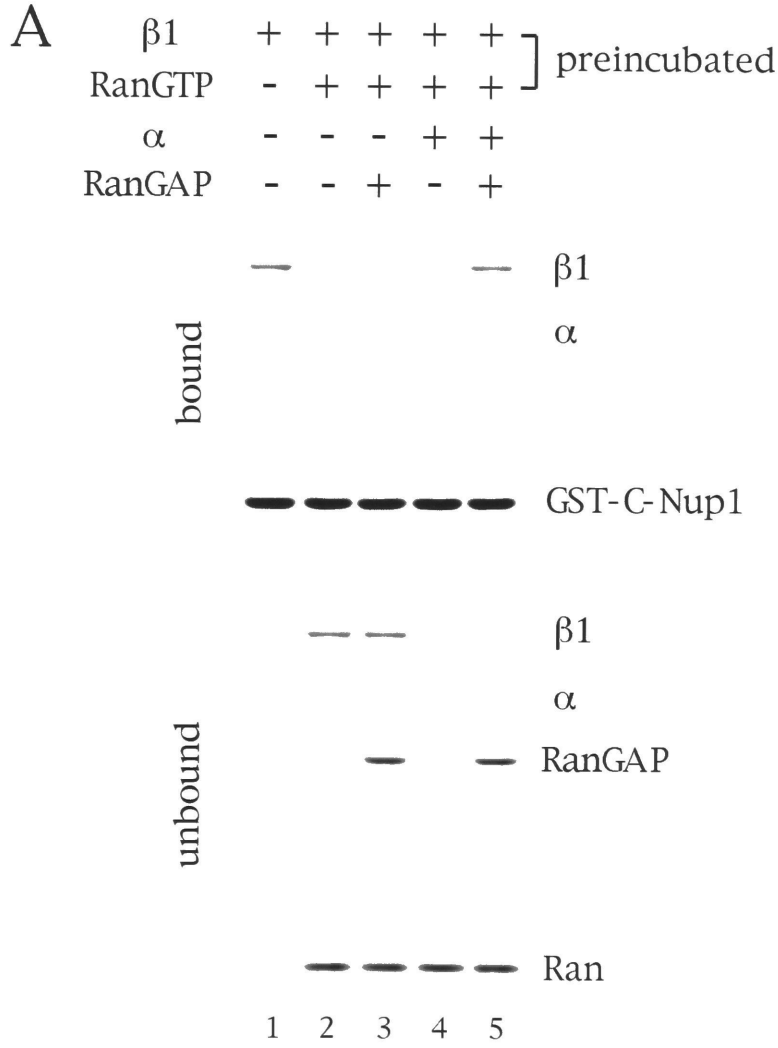
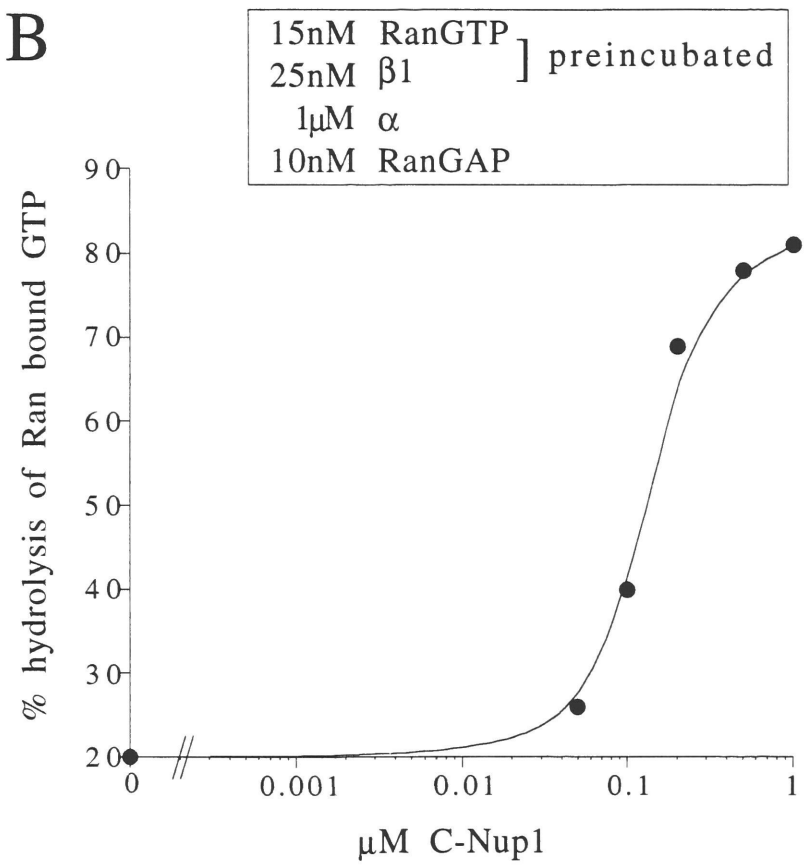

β1 + + + + +
RanGTP - + + - -
RanGDP - - - + +
β1
bound
GST-C-Nup1
β1
unbound
Ran
1 2 3 4 5

Fig. 6 The RanGTP/karyopherin β1 complex is disassembled in the presence of karyopherin α, RanGAP, and C-Nup1.

A , immobilized GST-C-Nup1 (1.25 μM final concentration) was incubated with 0.15 μM karyopherin β1 (lane 1) or 0.15 μM karyopherin β1 that had been preincubated with 1 μM RanGTP for 15 min at 4°C (lanes 2-5). RanGTP was prepared as described in the Experimental Procedures. Reactions also contained 0.5 μM RanGAP (lane 3 and 5) and 0.25 μM karyopherin α (lane 4 and 5). Reactions were incubated for 45 min at 21°C and then for 15 min at 4°C. Bound and unbound fractions were analyzed by SDS-PAGE and Coomassie blue staining. *B,* GTP hydrolysis assays were performed as described in the Experimental Procedures. 15 nM Ran-[γ-^{32}P]GTP was preincubated with 25 nM karyopherin β1 for 10 min at 21°C. Then 10 nM RanGAP, 1 μM karyopherin α, and increasing amounts of C-Nup1 were added. Reactions were incubated for 20 min at 21°C. The extent of GTP hydrolysis was quantified as described in the Experimental Procedures.


A
β1
RanGTP
α
RanGAP
+ + + + +
- + + + +
- - - + +
- - + - +
preincubated
bound
β1
α
GST-C-Nup1
unbound
β1
α
RanGAP
Ran
1 2 3 4 5


B
15nM RanGTP
25nM β1
1μM α
10nM RanGAP
] preincubated
% hydrolysis of Ran bound GTP
90
80
70
60
50
40
30
20
0
0.001
0.01
0.1
1
μM C-Nup1

indicate that the RanGTP/karyopherin β1 complex is disrupted in the presence of karyopherin α, C-Nup1, and RanGAP, and that RanGTP is converted to RanGDP through GTP hydrolysis stimulated by RanGAP.

3.1.5 Ran bound GTP is hydrolyzed when RanGTP/karyopherin β1 complex is disassembled in the presence of karyopherin α, RanGAP and the C-terminus of Nup1

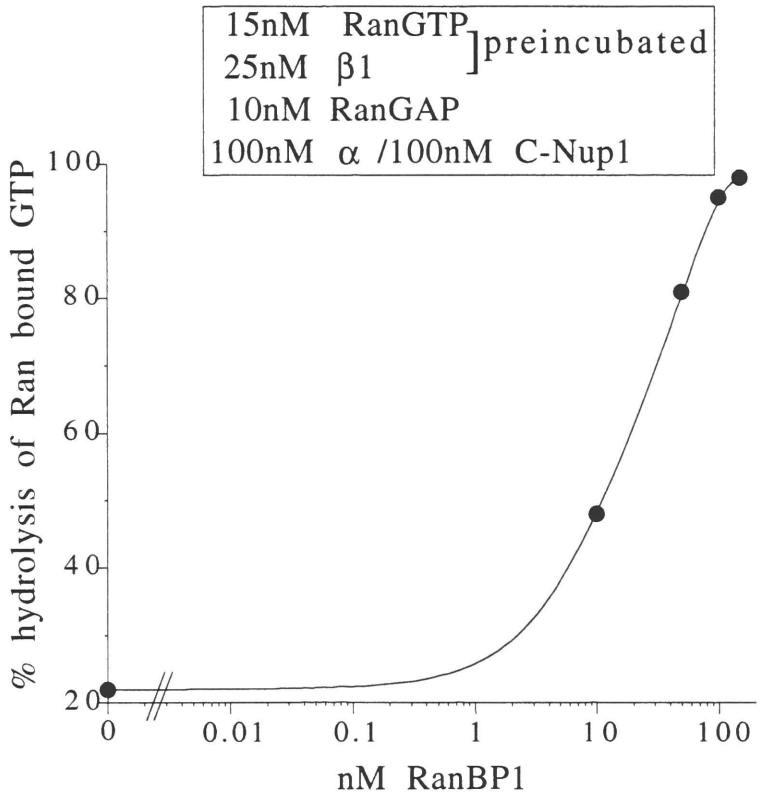
To test whether conversion of RanGTP to RanGDP occurred we measured GTP hydrolysis. RanGAP stimulated GTP hydrolysis by Ran was completely inhibited in the presence of karyopherin β1 (not shown)(82). However, addition of 1 μM karyopherin α and 10 nM RanGAP to a mixture of 15 nM RanGTP and 25 nM karyopherin β1 that had been preincubated, resulted in 20 % GTP hydrolysis (Fig. 6*B*). If 200 nM C-Nup1 was added to this reaction, 70 % of the Ran bound GTP was hydrolyzed, and if 1 μM C-Nup1 was added, 80 % of the Ran bound GTP was hydrolyzed. These results demonstrate that in the presence of karyopherin α RanGAP stimulates GTP hydrolysis by Ran that was bound to karyopherin β1, and that this reaction is stimulated by C-Nup1.

3.1.6 RanBP1 further stimulates disassembly of RanGTP/karyopherin β1 complex in the presence of karyopherin α, RanGAP and the C-terminus of Nup1

As the C-Nup1 stimulated conversion of RanGTP to RanGDP was only 80 %, we investigated whether RanBP1 could complete the reaction, as RanBP1 binds to RanGTP (74, 75) and enhances RanGAP stimulated GTP hydrolysis by Ran (75, 76, 86)(see Fig. 13). Addition of RanBP1 and RanGAP to a mixture of RanGTP and karyopherin β1 that had been preincubated did not promote GTP hydrolysis (data not shown). However, when RanBP1 and RanGAP were added together with karyopherin α and C-Nup1, GTP hydrolysis was greatly stimulated (Fig. 7). In a control reaction that contained 15 nM RanGTP and 25 nM karyopherin β1 that had been preincubated, 10 nM RanGAP, 100 nM karyopherin α, and 100 nM C-Nup1, 22 % of the Ran bound GTP was hydrolyzed (Fig. 7, closed symbols). When 50 nM RanBP1 was added, 80 % of the Ran bound GTP was hydrolyzed, and when 100 nM RanBP1 was added, 95 % of the Ran bound GTP was hydrolyzed. These results demonstrate that RanGTP/karyopherin β1 complex is fully disassembled in a reaction that requires karyopherin α and is stimulated by RanGAP, RanBP1, and the C-terminus of Nup1.

Fig. 7 RanBP1 stimulates GTP hydrolysis in the presence of karyopherin β1, RanGAP, karyopherin α, and C-Nup1.

GTP hydrolysis assays were performed as described in the Experimental Procedures. 15 nM Ran-[γ-^{32}P]GTP was preincubated with 25 nM karyopherin β1 for 10 min at 21°C. Then 100 nM karyopherin α, 100 nM C-Nup1, 10 nM RanGAP and increasing amounts of RanBP1 were added. Reactions were incubated for 20 min at 21°C. The extent of GTP hydrolysis was quantified as described in the Experimental Procedures.

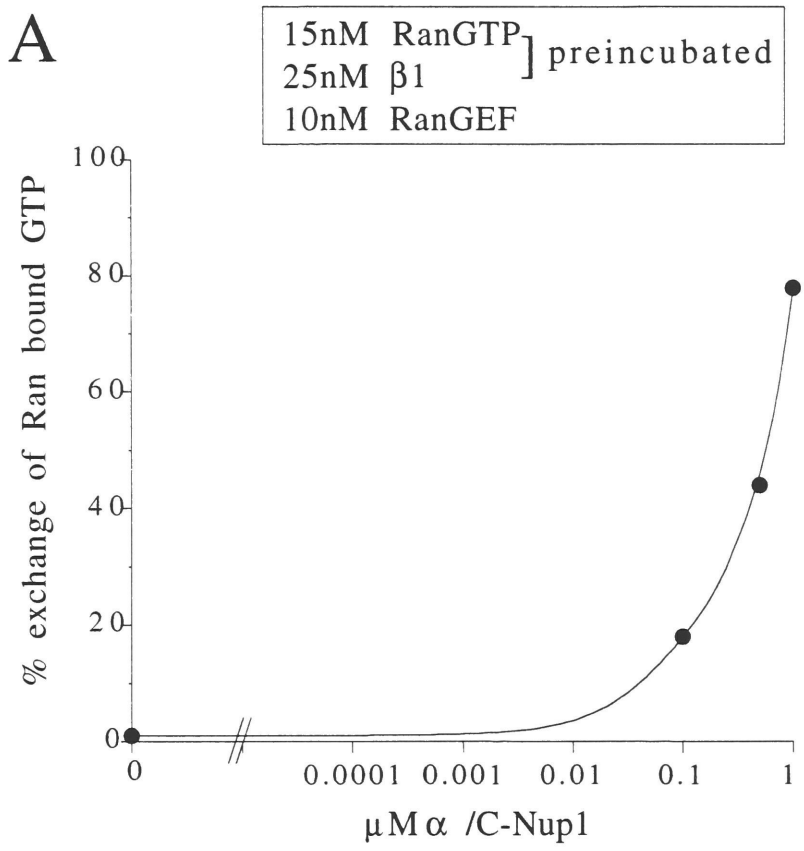

15nM RanGTP
25nM β1
]preincubated
10nM RanGAP
100nM α /100nM C-Nup1
% hydrolysis of Ran bound GTP
100
80
60
40
20
0
0.01
0.1
1
10
100
nM RanBP1

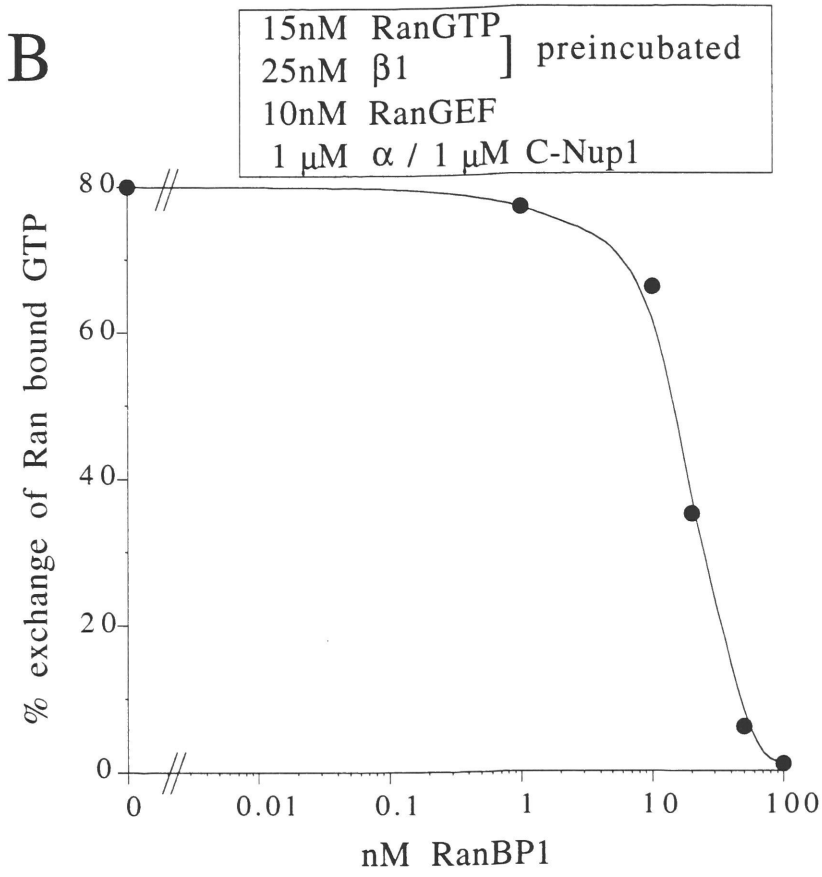
3.1.7 Disassembly of RanGTP/karyopherin β1 complex can also occur in the presence of karyopherin α, C-Nup1 and RanGEF

To determine if RanGAP is essential for the disassembly reaction we tested whether the nucleotide exchange factor for Ran, RanGEF, could replace RanGAP. RanGEF stimulates exchange of GDP and GTP on Ran with equal efficiency (33). Due to the higher concentration of GTP in the cell (32) it is generally assumed that RanGEF exchanges Ran bound GDP for GTP *in vivo* (33). Nevertheless, RanGEF can exchange Ran bound GTP for GDP *in vitro* if an excess of GDP is provided (33). Although recombinant RanGEF stimulated nucleotide exchange on Ran (not shown), it was incapable of stimulating exchange when RanGTP was bound to karyopherin β1 (Fig. 8*A*)(72). However, exchange of Ran bound GTP occurred when 10 nM RanGEF, 200 μM GDP, 200 μM GTP, and increasing amounts of karyopherin α and C-Nup1 were added to 15 nM RanGTP and 25 nM karyopherin β1 that had been preincubated (Fig. 8*A*). GTP exchange reached 80 % in the presence of 1 μM karyopherin α and 1 μM C-Nup1. Addition of C-Nup1 in the absence of karyopherin α did not result in GTP exchange, whereas addition of 1 μM karyopherin α in the absence of C-Nup1 promoted exchange of 25 % of the Ran bound GTP (data not shown). We also tested the effect of RanBP1 on this

Fig. 8 RanGEF can replace RanGAP in disassembly of RanGTP/karyopherin β1 complex. RanGEF is inhibited by RanBP1.

Guanine nucleotide exchange assays were performed as described in the Experimental Procedures. 15 nM Ran-[α-^{32}P]GTP was preincubated with 25 nM karyopherin β1 for 10 min at 21°C. *A,* 10 nM RanGEF, 200 μM GDP, 200 μM GTP and increasing amounts of karyopherin α and C-Nup1 were added as indicated. *B,* 10 nM RanGEF, 200 μM GDP and 200 μM GTP were added in the presence of 1 μM karyopherin α, 1 μM C-Nup1 and increasing amounts of RanBP1. Reactions were incubated for 20 min at 21°C. The extent of GTP exchange was quantified as described in the Experimental Procedures.


A
15nM RanGTP
25nM β1
10nM RanGEF
preincubated
% exchange of Ran bound GTP
100
80
60
40
20
0
0
0.0001
0.001
0.01
0.1
1
μM α /C-Nup1

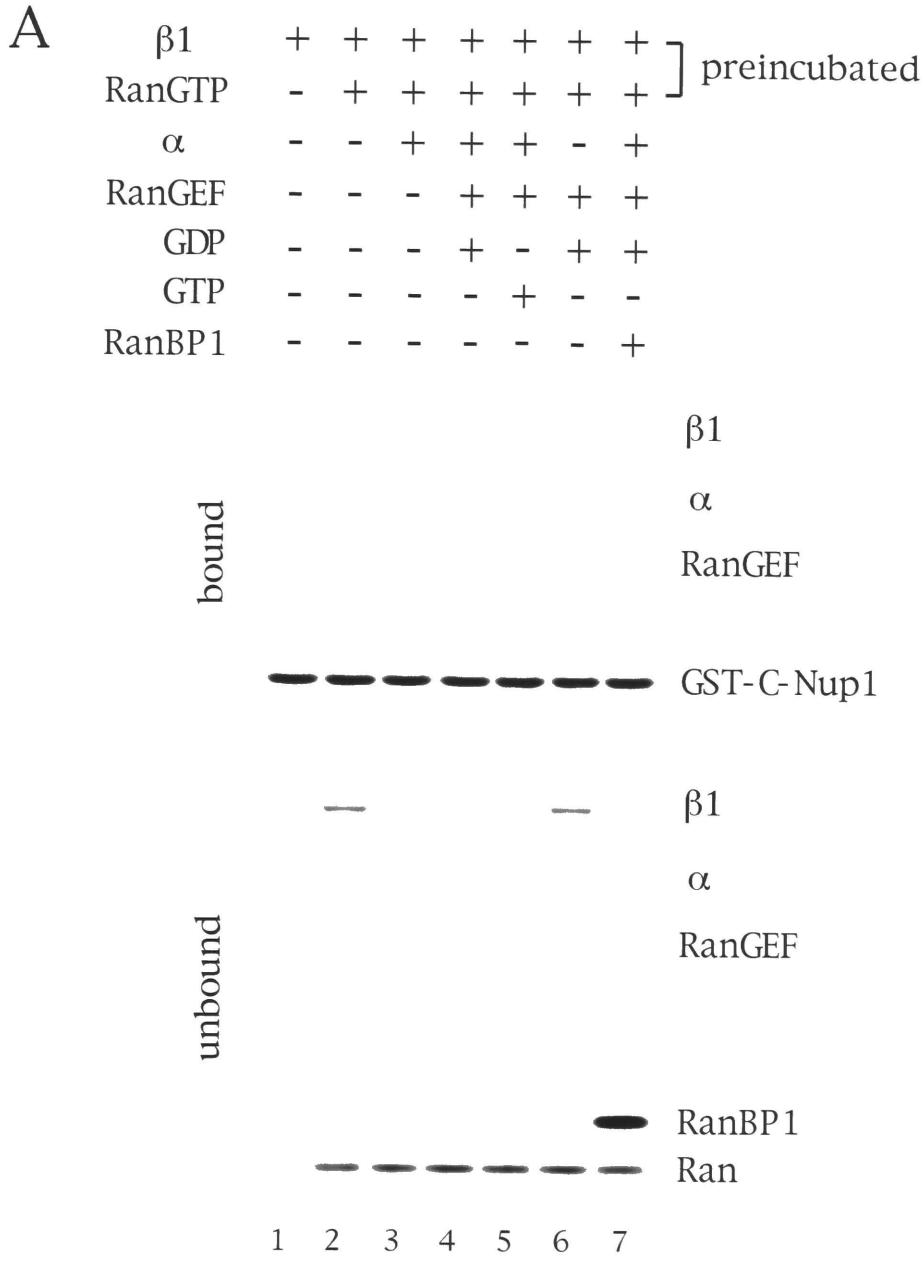

B
15nM RanGTP
25nM β1
] preincubated
10nM RanGEF
1 μM α / 1 μM C-Nup1
% exchange of Ran bound GTP
80
60
40
20
0
0
0.01
0.1
1
10
100
nM RanBP1

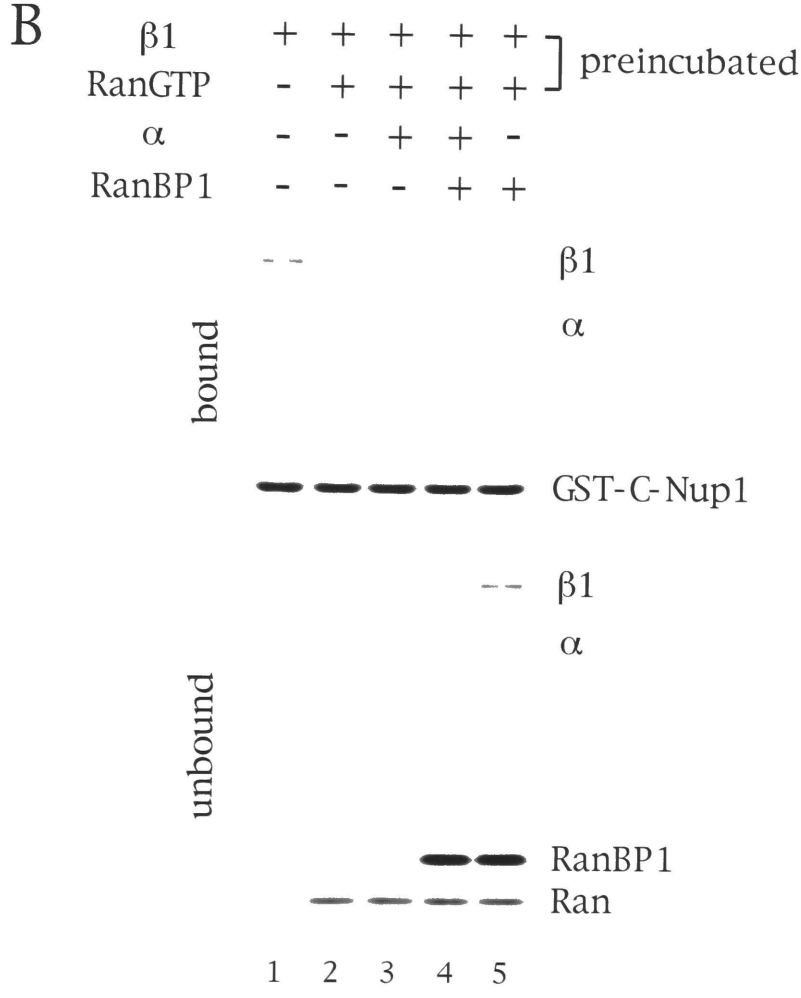
reaction. 50 nM RanBP1 inhibited exchange of Ran bound GTP when added to a reaction that contained 10 nM RanGEF, 200 μM GDP, 200 μM GTP, 1 μM karyopherin α, 1 μM C-Nup1, and 15 nM RanGTP and 25 nM karyopherin β1 that had been preincubated (Fig. 8*B*). This result is consistent with previous reports on the inhibitory effect of RanBP1 on the exchange of Ran bound GTP when karyopherin β1 is absent (75, 86).

To further analyze the effect of RanGEF and RanBP1 on the disassembly of RanGTP/karyopherin β1 complex, we carried out solution binding assays similar to those described in Fig. 6*A*. As expected, RanGEF could replace RanGAP (Fig. 9*A*) when an excess of GDP (lane 4), but not GTP (lane 5), was present in addition to karyopherin α and C-Nup1. Karyopherin α was required, as RanGEF and GDP alone did not promote karyopherin β1 binding to C-Nup1 (lane 6). This result suggests that RanGTP is accessible to RanGAP, or RanGEF, after the RanGTP/karyopherin β1 complex is disassembled by the combined action of karyopherin α and C-Nup1. Surprisingly, RanGEF bound to the C-terminus of Nup1 (lanes 4-7).

Fig. 9 RanBP1 stimulates RanGTP/karyopherin β1 disassembly in the presence of karyopherin α, C-Nup1, and in the presence and absence of RanGEF.

A, immobilized GST-C-Nup1 (1.25 μM final concentration) was incubated with 0.15 μM karyopherin β1 (lane 1) or 0.15 μM karyopherin β1 that had been preincubated with 1 μM RanGTP for 15 min at 4°C (lanes 2-7). RanGTP was prepared as described in the Experimental Procedures. Reactions also contained 0.25 μM karyopherin α (lanes 3-5, 7), 0.2 μM RanGEF (lanes 4-7), 250 μM GDP (lanes 4,6 and 7), 250 μM GTP (lane 5) and 1.6 μM RanBP1 (lane 7). Reactions were incubated for 45 min at 21°C and then for 15 min at 4°C. Bound and unbound fractions were analyzed by SDS-PAGE and Coomassie blue staining. *B,* immobilized GST-C-Nup1 (1.25 μM final concentration) was incubated with 0.15 μM karyopherin β1 (lane 1) or 0.15 μM karyopherin β1 that had been preincubated with 1 μM RanGTP for 15 min at 4°C (lanes 2-5). Then 0.25 μM karyopherin α (lane 3 and 4) and 1.6 μM RanBP1 (lanes 4 and 5) were added. Reactions were incubated for 1 h at 4°C. Bound and unbound fractions were analyzed by SDS-PAGE and Coomassie blue staining.


A
β1 + + + + + + +
RanGTP - + + + + + +
α - - + + + - +
RanGEF - - - + + + +
GDP - - - + - + +
GTP - - - - + - -
RanBP1 - - - - - - +
preincubated
bound
β1
α
RanGEF
GST-C-Nup1
unbound
β1
α
RanGEF
RanBP1
Ran
1 2 3 4 5 6 7


B
β1
RanGTP
α
RanBP1
+ + + + +
- + + + +
- - + + -
- - - + +
preincubated
β1
α
bound
GST-C-Nup1
β1
α
unbound
RanBP1
Ran
1 2 3 4 5

3.1.8 RanBP1 directly stimulates disassembly of RanGTP/karyopherin β1 complex in the presence of karyopherin α and C-Nup1, and in the absence of RanGAP

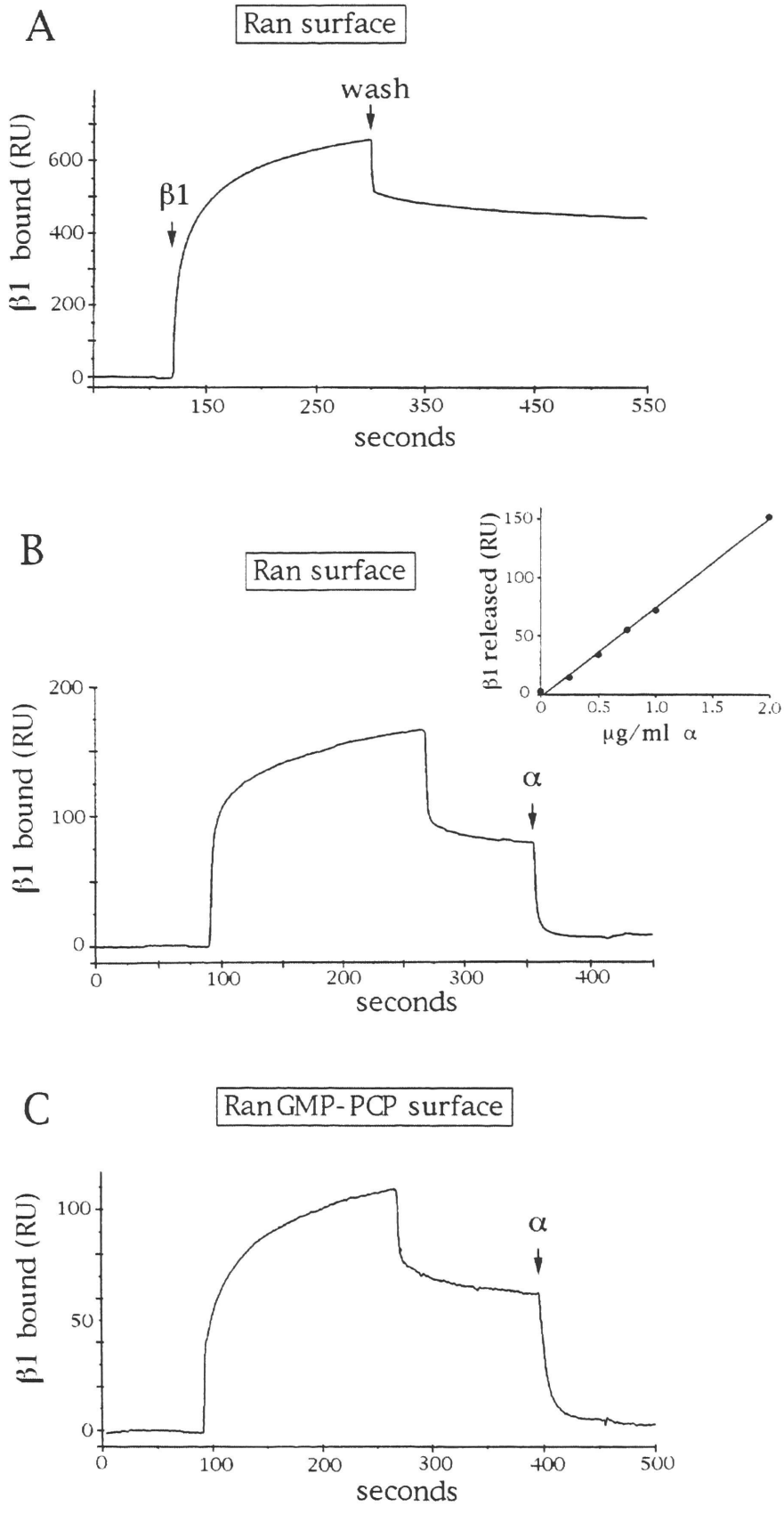
We then tested whether inhibition of RanGEF function by RanBP1 had an effect on RanGTP/karyopherin β1 disassembly. Unexpectedly, disassembly occurred even in the presence of RanBP1 (Fig. 9*A*, compare lane 7 to lane 4). This surprising result suggests that RanBP1 can directly stimulate disassembly of the RanGTP/karyopherin β1 complex in the presence of karyopherin α and C-Nup1. Indeed RanBP1 stimulated disassembly in the presence of karyopherin α and C-Nup1 (Fig. 9*B*, lane 4), but not in the absence of karyopherin α (lane 5). Thus karyopherin α is required to disrupt the RanGTP/karyopherin β1 complex in the presence of RanBP1 and C-Nup1. Neither RanBP1 nor RanGTP bound to the karyopherin α/β1/C-Nup1 complex (not shown). These results suggest that RanBP1 stimulates disassembly of the RanGTP/karyopherin β1 complex by preventing reformation of the complex after it has been disassembled in the presence of karyopherin α and C-Nup1. RanBP1 may accomplish this task by sequestering RanGTP that has been released from karyopherin β1, since RanBP1 binds RanGTP (not shown)(74, 75, 86).

3.1.9 Karyopherin α dissociates karyopherin β1 from RanGTP

To analyze in detail the mechanism of RanGTP/karyopherin β1 disassembly we used surface plasmon resonance (83), which allows a direct measurement of on/off rates of protein-protein interactions. Ran containing ~ 40 % GTP and ~ 60 % GDP was immobilized on a CM5 sensor chip by amine coupling as described in the Experimental Procedures. 100 μg/ml karyopherin β1 was injected for 3 min at a flow rate of 10 μl/min over the Ran surface which typically resulted in binding of 500 resonance units (RU) of karyopherin β1 (Fig. 10*A*). This was followed by a wash-out phase of 3 min to monitor karyopherin β1 dissociation. We then examined whether the dissociation rate of the RanGTP/karyopherin β1 complex increases when other proteins are added during the wash-out phase. For these experiments 100-200 RU of karyopherin β1 were bound to the Ran surface, dissociation was allowed to proceed for 1 min, and solutions containing different factors were injected for 1 min. Strikingly, injection of karyopherin α caused the release of all karyopherin β1 from RanGTP (Fig. 10*B*). The amount released was dependent on the concentration of karyopherin α injected with a linear relation between released karyopherin β1 and the concentration of karyopherin α (0-2 μg/ml of karyopherin α)(Fig. 10*B*, insert).

Fig. 10 Karyopherin α releases karyopherin β1 from RanGTP.

A, Ran that was ~ 40 % GTP and ~ 60 % GDP bound was immobilized on a CM5 sensor chip as described in the Experimental Procedures. Injection of 100 μg/ml karyopherin β1 resulted in binding of 500 RU to the Ran surface. *B*, Ran was immobilized as in *A*, and 90 RU of karyopherin β1 were bound to the Ran surface. 1 μg/ml karyopherin α was injected for 1 min during the wash-out phase. The insert shows release of karyopherin β1 by various concentrations of karyopherin α. The amount of karyopherin β1 released was plotted against the concentration of karyopherin α used. *C*, Ran that was ~ 39 % GMP-PCP, ~ 60 % GDP and < 1 % GTP bound was immobilized as described in the Experimental Procedures. 70 RU of karyopherin β1 were bound to the surface and 2 μg/ml karyopherin α was injected for 1 min during the wash-out phase.


A
Ran surface
wash
β1
β1 bound (RU)
600
400
200
0
150
250
350
450
550
seconds
B
Ran surface
β1 released (RU)
150
100
50
0
0
0.5
1.0
1.5
2.0
μg/ml α
α
β1 bound (RU)
200
100
0
0
100
200
300
400
seconds
C
Ran GMP-PCP surface
α
β1 bound (RU)
100
50
0
0
100
200
300
400
500
seconds

The apparent rate of RanGTP/karyopherin β1 dissociation in the presence of saturating concentrations of karyopherin α was faster than the detection limit of the BIAcore instrument (> 0.1 s^{-1}); hence, stimulation of RanGTP/karyopherin β1 dissociation by karyopherin α could not be measured directly. The rate of RanGTP/karyopherin β1 dissociation in the absence of karyopherin α was calculated to be 4.5 x 10^{-4} s^{-1} based on the conditions of these experiments. We therefore estimate that karyopherin α stimulates the dissociation rate of the RanGTP/karyopherin β1 complex by at least three orders of magnitude. RanGAP did not increase the amount of karyopherin β1 released in the presence of limiting amounts of karyopherin α (not shown). This result is in agreement with the notion that RanGAP interacts with RanGTP only after its release from karyopherin β1.

3.1.10 GTP hydrolysis on Ran is not required for dissociation of karyopherin β1 from the RanGTP/karyopherin β1 complex by karyopherin α

To test whether intrinsic GTP hydrolysis by Ran is a prerequisite for karyopherin α induced dissociation of karyopherin β1 we used RanGMP-PCP instead of RanGTP; GMP-PCP is a non-hydrolyzable analog of GTP. Ran was

incubated with GMP-PCP as described in the Experimental Procedures to obtain Ran that was ~ 39 % GMP-PCP, ~ 60 % GDP, and less than 1 % GTP bound. RanGMP-PCP was immobilized on a CM5 sensor chip as described for Ran. Karyopherin β1 bound to RanGMP-PCP with the same apparent kinetics as to RanGTP under these conditions (compare Fig. 10*C* to 10*A*). When karyopherin α was injected during the wash-out phase, all the karyopherin β1 was released (Fig. 10*C*). The release of karyopherin β1 from RanGMP-PCP showed the same dependence on the concentration of karyopherin α as release of karyopherin β1 from RanGTP (not shown). This result demonstrates that GTP hydrolysis is not required for the karyopherin α dependent dissociation of RanGTP from karyopherin β1.

3.1.11 The C-terminus of Nup1 prevents rebinding of karyopherin β1 to RanGTP by sequestering the newly formed karyopherin α/β1 complex

We also used surface plasmon resonance to investigate the role of C-Nup1 in the disassembly of the RanGTP/karyopherin β1 complex. C-Nup1 did not release karyopherin β1 from RanGTP when injected during the wash-out phase (not shown). Also, coinjection of C-Nup1 with karyopherin α did not stimulate

release over the levels seen with karyopherin α alone (not shown). These results were surprising as C-Nup1 greatly stimulates the disruption of the RanGTP/karyopherin β1 complex by karyopherin α, as judged by the GTP hydrolysis assay (Fig. 6*B*), yet does not stimulate RanGAP activity directly (not shown). To understand the role of C-Nup1 in the RanGTP/karyopherin β1 disassembly reaction, we compared the GTP hydrolysis and surface plasmon resonance experiments. In the surface plasmon resonance experiments the disassembly reaction is monitored in real time and not at equilibrium as in the GTP hydrolysis assay. In the GTP hydrolysis assay the released karyopherin β1 may rebind to RanGTP before RanGAP stimulates GTP hydrolysis, whereas in the surface plasmon resonance experiment the released karyopherin β1 is removed by constant flow and cannot rebind to RanGTP. In surface plasmon resonance rebinding during the wash-out phase is significant only, when high density surfaces and low flow rates are used (87). As there was only ~ 160 RU of RanGTP immobilized on the surface, and there was no change in the RanGTP/karyopherin β1 dissociation rate at flow rates of up to 30 µl/min, we assume that rebinding of karyopherin β1 to RanGTP did not occur. To test whether C-Nup1 affects rebinding of karyopherin β1 to RanGTP, we coinjected

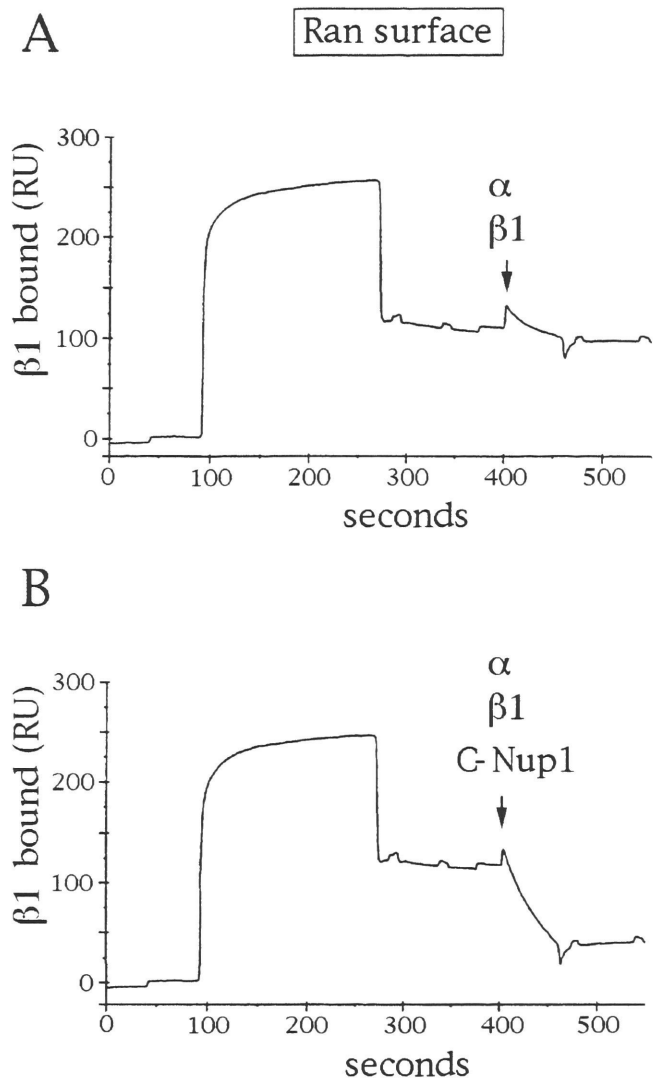
karyopherin β1 during the wash-out phase; this retards the diffusion of dissociated karyopherin β1 from the Ran surface and may promote rebinding of karyopherin β1 before it is removed by the wash. When 5 nM karyopherin β1 was coinjected with 35 nM karyopherin α (2 μg/ml), release of karyopherin β1 was completely inhibited (Fig. 11*A*). Strikingly, release of karyopherin β1 from the RanGTP surface was restored when 35 nM C-Nup1 was coinjected with 35 nM karyopherin α and 5 nM karyopherin β1 (Fig. 11*B*). This result indicates that C-Nup1 sequesters karyopherin α/β1 and prevents reformation of the RanGTP/karyopherin β1 complex.

3.1.12 The nucleoporins NSP1, Nup2, Nup36 and a fragment of Nup1 containing its FXFG-peptide repeat region stimulate disassembly of RanGTP/karyopherin β1 complex in the presence of karyopherin α and RanGAP

As yeast karyopherin α/β1 also binds to the FXFG repeat region of Nup1(77), Nup2 (77) and Nup36 (46), we tested whether these nucleoporins or fragments thereof could replace C-Nup1 in the karyopherin α-mediated disassembly of the RanGTP/karyopherin β1 complex using the GTP hydrolysis

Fig. 11 The C-terminus of Nup1 sequesters karyopherin α/β1 and prevents reformation of the RanGTP/karyopherin β1 complex.

A, 120 RU of karyopherin β1 were bound to a Ran surface prepared with Ran containing ~ 40 % GTP and ~ 60 % GDP as described in the Experimental Procedures. During the wash-out phase a solution containing 35 nM karyopherin α and 5 nM karyopherin β1 was injected for 1 min. *B,* 120 RU of karyopherin β1 were bound to the Ran surface as in *A*. During the wash-out phase a solution containing 35 nM C-Nup1, 5 nM karyopherin β1, and 35 nM karyopherin α was injected for 1 min.


A
Ran surface
β1 bound (RU)
300
200
100
0
α
β1
0
100
200
300
400
500
seconds
B
β1 bound (RU)
300
200
100
0
α
β1
C-Nup1
0
100
200
300
400
500
seconds

assay (for a schematic view of the constructs see Fig. 12*A*). In addition we tested the nucleoporin NSP1. As a control, addition of 0.75 μM karyopherin α and 10 nM RanGAP to 15 nM RanGTP and 25 nM karyopherin β1 that had been preincubated resulted in 10 % hydrolysis of the Ran bound GTP (Fig. 12*B*). Addition of 1 μM C-Nup1 to this mixture resulted in 84 % hydrolysis. When 1 μM Nup1*, a fragment of Nup1 containing its FXFG-peptide repeat region, was added instead of C-Nup1, 33 % of the Ran bound GTP was hydrolyzed. Likewise, addition of 1 μM Nup36 resulted in 36 % hydrolysis, 1 μM Nup2 resulted in 67 %, and 1 μM NSP1 resulted in 91 % hydrolysis. Interestingly, addition of Nup2*, a fragment of Nup2 containing only its FXFG-peptide repeat region, resulted only in 15 % hydrolysis. These findings demonstrate that the FXFG repeat region of Nup1, full length Nup2, Nup36, and NSP1 can efficiently stimulate the RanGTP/karyopherin β1 disassembly reaction.

3.1.13 Nup36 stimulates RanGAP induced GTP hydrolysis on RanGTP

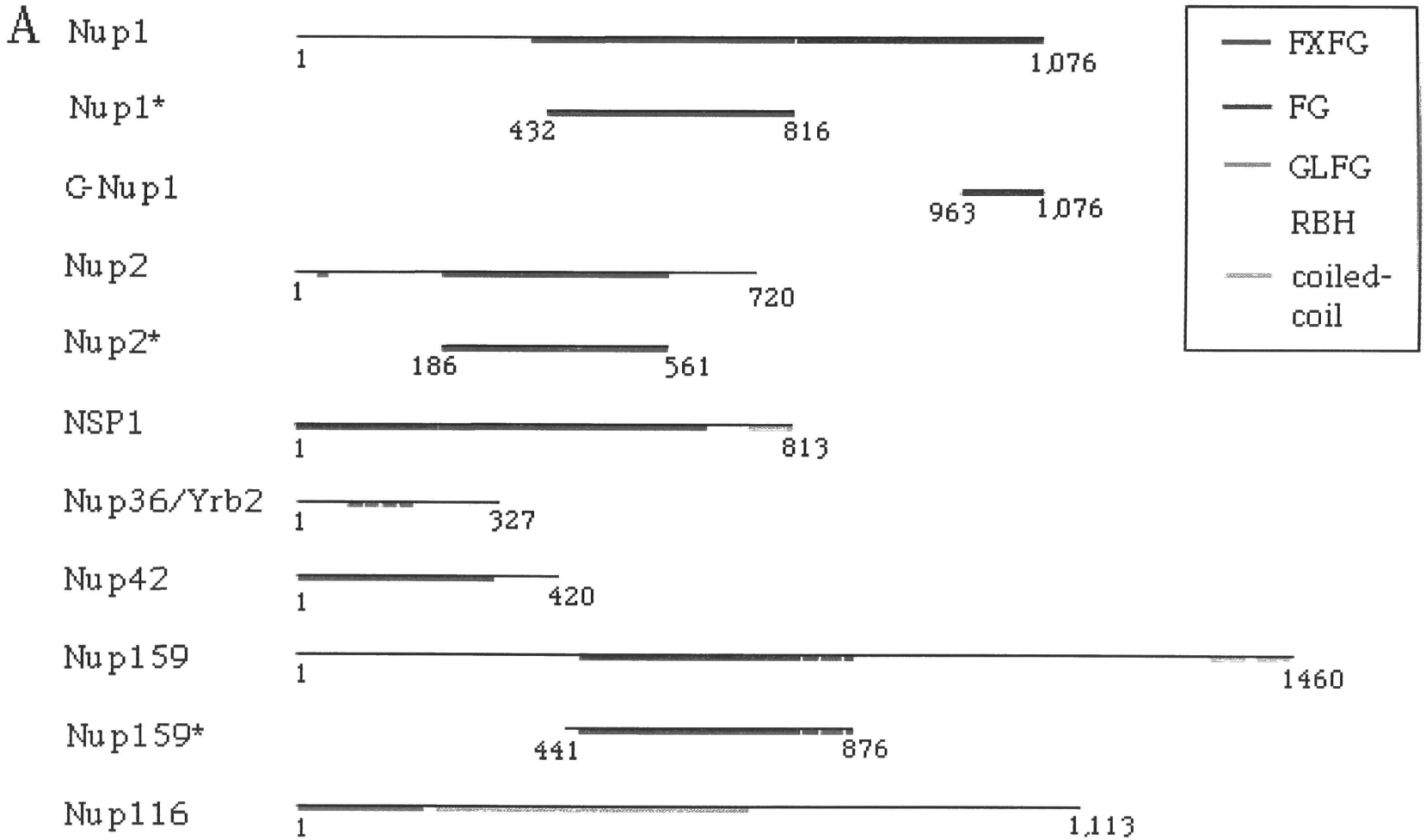
Nup36, in addition to binding karyopherin α/β1 (46), also binds RanGTP (Floer, M., Nehrbass, U. and Blobel, G., unpublished observation)(88) presumably through a RBH-domain in its C-terminus (46, 88-90). We find that Nup36

Fig. 12 RanGTP/karyopherin β1 disassembly is promoted by different fragments of nucleoporins that bind karyopherin α/β1.

A Schematic view of the Nup constructs used in the disassembly reaction. *B* GTP hydrolysis assays were performed as described in the Experimental Procedures. 15 nM Ran-[γ-^{32}P]GTP was preincubated with 25 nM karyopherin β1 for 10 min at 21°C. Then 10 nM RanGAP and 0.75 μM karyopherin α were added in the presence or absence of 1 μM of the nucleoporin fragments as indicated. Reactions were incubated for 20 min at 21°C. The extent of GTP hydrolysis was quantified as described in the Experimental Procedures.

Nup1* signifies a fragment of Nup1 containing its FXFG-peptide repeat region.

Nup2* signifies a fragment of Nup2 containing its FXFG-peptide repeat region.


A
Nup1
1
1,076
Nup1*
432
816
G-Nup1
963
1,076
Nup2
1
720
Nup2*
186
561
NSP1
1
813
Nup36/Yrb2
1
327
Nup42
1
420
Nup159
1
1460
Nup159*
441
876
Nup116
1
1,113
FXFG
FG
GLFG
RBH
coiled-coil

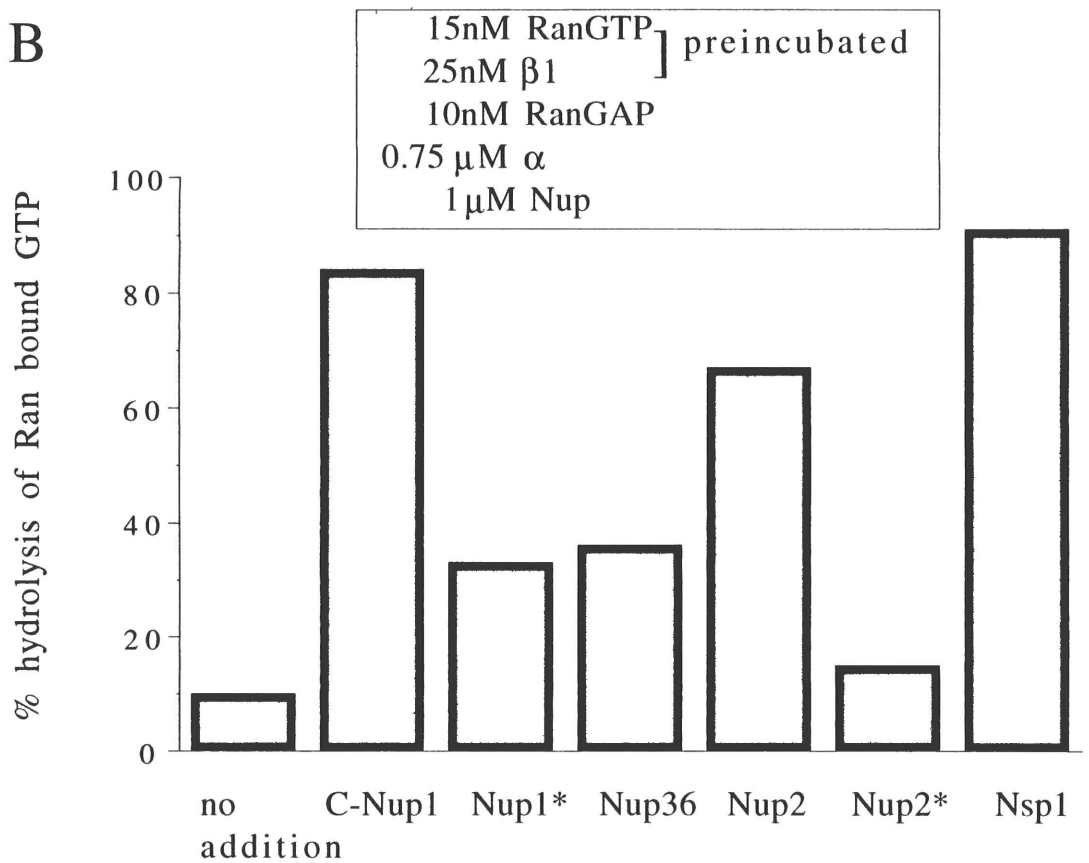
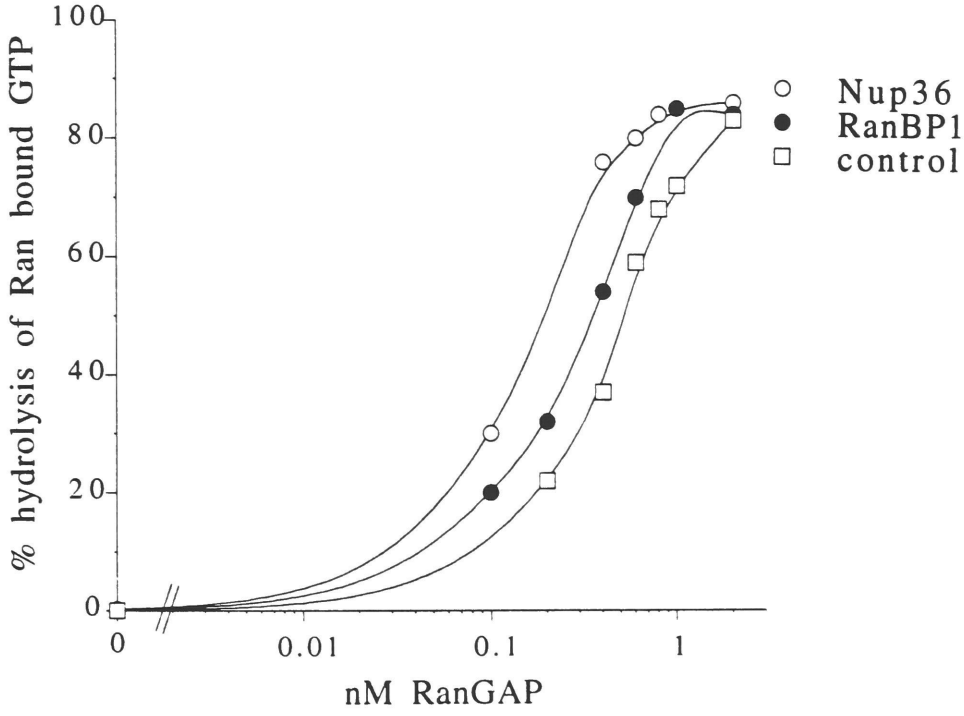

B
15nM RanGTP
25nM β1
preincubated
10nM RanGAP
0.75 μM α
1 μM Nup
% hydrolysis of Ran bound GTP
100
80
60
40
20
0
no addition
C-Nup1
Nup1*
Nup36
Nup2
Nup2*
Nsp1

Fig. 13 Nup36 stimulates GTP hydrolysis in the presence of RanGAP.

GTP hydrolysis assays were conducted as described in the Experimental Procedures. 15 nM Ran-[γ-^{32}P]GTP was incubated for 10 min at 21°C in the presence of 1 μM RanBP1 (closed circles), 1 μM Nup36 (open squares) or no addition (open circles). Then, increasing amounts of RanGAP were added followed by incubation for 20 min at 21°C.


100
80
60
40
20
0
% hydrolysis of Ran bound GTP
0
0.01
0.1
1
nM RanGAP
Nup36
RanBP1
control

can stimulate GAP activity in a manner similar to RanBP1 (Fig. 13)(88). Addition of 1 μM Nup36 (open squares) resulted in greater stimulation of GAP activity than addition of 1 μM RanBP1 (closed circles). This result suggests that Nup36 functions in the RanGTP/karyopherin β1 disassembly reaction by stimulating conversion of RanGTP to RanGDP via GTP hydrolysis in the presence of RanGAP, as well as by sequestering karyopherin α/β1.

3.2 Discussion

Our data here show that the RanGAP stimulated GTP hydrolysis of RanGTP is inhibited by karyopherin β1 in a concentration dependent manner (Fig. 2 and 3). In gel filtration experiments RanGTP and karyopherin β1 were found to form a stoichiometric complex (Fig. 4). We suggest that binding of RanGTP to karyopherin β1 renders RanGTP inaccessible to RanGAP. RanGTP binding to karyopherin β1 does not affect the slow intrinsic GTPase activity of Ran as no differences in the rates of GTP hydrolysis could be detected during a one hour incubation of RanGTP either in the absence or presence of karyopherin β1 (data not shown).

Unlike Ras which has an affinity for GTP that is about one order of magnitude higher than that for GDP (91), Ran has a 10-fold higher affinity for GDP than it has for GTP (33). Up to 80 % of the cellular Ran is thought to be located in the nucleus (26), whereas RanGAP is thought to be located in the cytoplasm (38). Hence, the cytoplasmic RanGAP is likely to keep the cytoplasmic concentration of RanGTP low, because cytoplasmic RanGTP would be detrimental for nuclear import. It would dissociate the karyopherin heterodimer in the cytoplasm, associate with karyopherin β1 and thereby prevent targeting of an

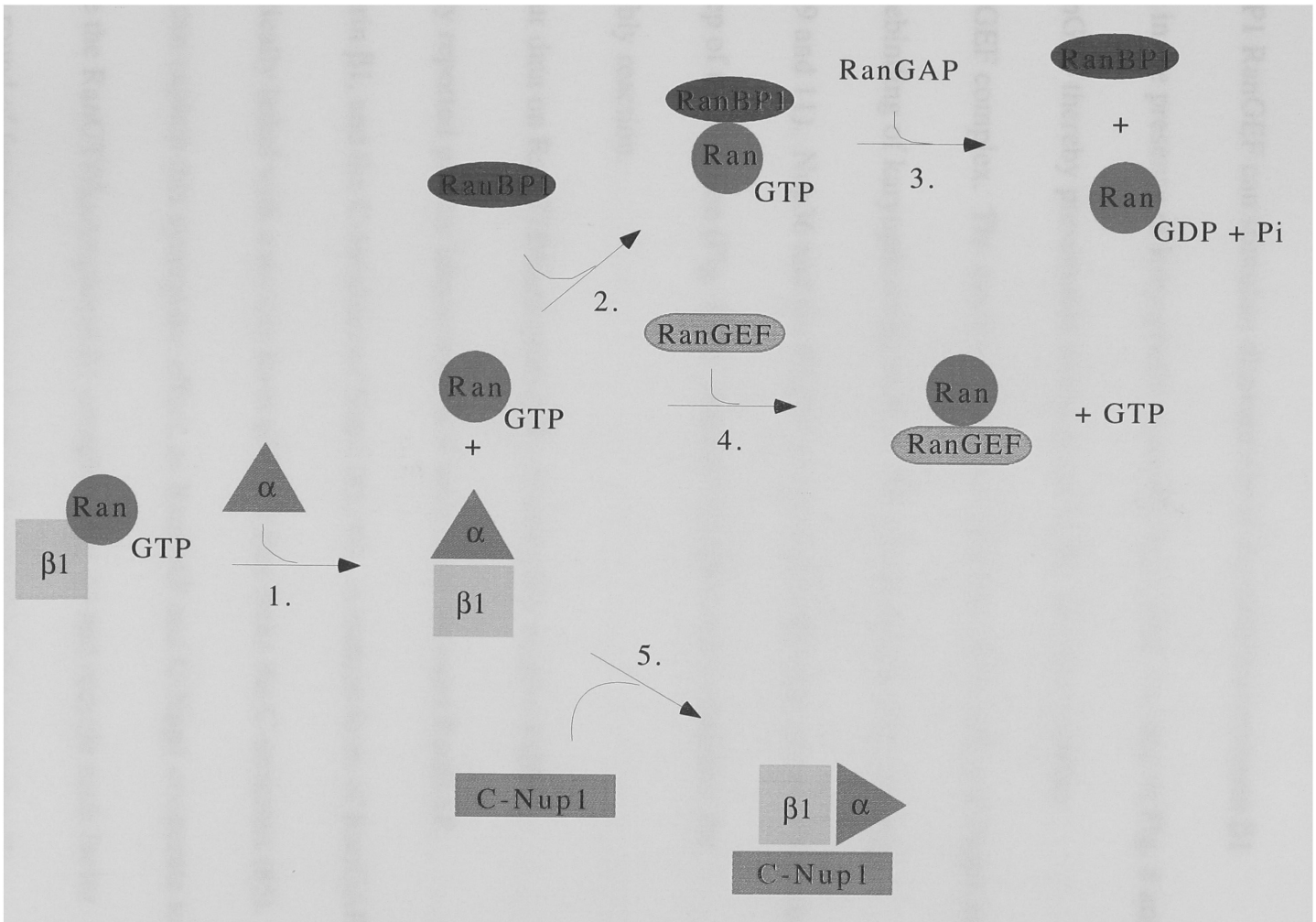
NLS-protein to the nuclear pore complex (77).

The formation of a complex between RanGTP and karyopherin β1 that renders RanGTP inaccessible to RanGAP is reminiscent of the formation of a complex of RasGTP with its downstream effector Raf kinase that has been proposed to render RasGTP inaccessible to RasGAP (92, 93). Ras/Raf interaction is thought to be terminated through intrinsic GTP hydrolysis by Ras, resulting in the release of Raf kinase (94). Similarly, intrinsic GTP hydrolysis by Ran could result in dissociation of Ran from karyopherin β1, as the affinity of the latter for RanGDP is lower than for RanGTP (Fig. 4). However, the intrinsic rate of GTP hydrolysis by Ran is rather low (95). Therefore, dissociation of RanGTP/karyopherin β1 complex may occur via a different mechanism in the cell.

Our results suggest a model for RanGTP/karyopherin β1 disassembly (Fig. 14). First, karyopherin β1 is released from RanGTP by karyopherin α in a reaction that does not require GTP hydrolysis (Fig. 14, step 1; Fig. 10). RanGTP is then bound by RanBP1, which prevents reformation of the RanGTP/karyopherin β1 complex in the presence of C-Nup1 and karyopherin α (Fig. 14, step 2; Fig. 9*B*). RanGTP bound to RanBP1 has a higher affinity for RanGAP than free RanGTP, so that conversion of RanGTP to RanGDP is enhanced by RanBP1 (Fig.

Fig. 14 Model for the mechanism of RanGTP/karyopherin β1 complex disassembly .

Karyopherin α releases RanGTP from karyopherin β1 (step 1). RanBP1 then binds RanGTP and prevents reformation of the RanGTP/karyopherin β1 complex in the presence of C-terminus of Nup1 (C-Nup1) and karyopherin α (step 2). Subsequently, RanGTP is converted to RanGDP via GTP hydrolysis in the presence of RanGAP (step 3). Alternatively, RanGEF can bind to RanGTP, which results in release of GTP and formation of a Ran/RanGEF complex (step 4). The newly formed karyopherin α/β1 binds to the C-Nup1 and inhibits rebinding of karyopherin β1 to RanGTP (step 5).


RanGAP
RanBP1
RanBP1
Ran
GTP
+
Ran
GDP + Pi
3.
RanBP1
2.
RanGEF
Ran
GTP
+
4.
Ran
RanGEF
+ GTP
α
Ran
β1
GTP
α
β1
1.
5.
C-Nup1
β1
α
C-Nup1

14, step 3; Fig. 7 and 13)(75, 76, 86). RanBP1 binding to RanGTP also prevents interaction of RanGTP with RanGEF (Fig. 8*B*)(75, 86). However, in the absence of RanBP1 RanGEF can stimulate disassembly of RanGTP/karyopherin β1 complex in the presence of karyopherin α and C-Nup1 (Fig. 14, step 4; Fig. 8 and 9*A*). RanGEF thereby presumably forms a nucleotide free intermediate Ran/RanGEF complex. The newly formed karyopherin α/β1 binds to C-Nup1 and renders rebinding of karyopherin β1 to RanGTP less favorable (Fig. 14, step 5; Fig. 6A, 9 and 11). Nup36 and the FXFG repeat region of Nup1 can also function in this step of the reaction (Fig. 12*B*). Additional factors may modulate the disassembly reaction.

Our data on RanGTP/karyopherin β1 disassembly *in vitro* explain previously reported genetic interactions in *S. cerevisiae* between RanGAP, karyopherin β1, and the C-terminus of Nup1 (85, 96). A mutant form of RanGAP is synthetically lethal with a mutant form of Nup1 that lacks the C-terminus (85). Our data can explain this synergistic effect, as RanGAP and C-Nup1 cooperate to dissociate the RanGTP/karyopherin β1 complex (Fig. 6) and recycle each factor for a new round of function. A mutant form of RanGAP is also synthetically lethal with a mutant form of karyopherin β1 (96). This genetic interaction can be

explained as well, since our data show that RanGAP promotes recycling of karyopherin β1 by stimulating RanGTP/karyopherin β1 disassembly (Fig. 6).

RanGTP/karyopherin β1 complex is disassembled by karyopherin α through active release, as karyopherin α increases the rate of RanGTP/karyopherin β1 dissociation (Fig. 10*B*). Release of karyopherin β1 from RanGTP is linearly dependent on the concentration of karyopherin α (Fig. 10*B*, insert). We estimate that karyopherin α stimulates RanGTP/karyopherin β1 dissociation by at least three orders of magnitude. Release of RanGTP from karyopherin β1 presumably occurs through formation of an intermediate RanGTP/karyopherin β1/α complex, followed by dissociation of RanGTP. We did not detect this intermediate complex possibly because the displacement reaction is too fast to be resolved using surface plasmon resonance. The proposed displacement mechanism is supported by data, which demonstrate that RanGTP and karyopherin α have partially overlapping binding sites on karyopherin β1 (97-99). Thus karyopherin α could interact with the RanGTP/karyopherin β1 complex through that part of its binding site on karyopherin β1 that is not occupied by RanGTP; this interaction might then displace RanGTP from the overlapping site. The displacement reaction is reversible as RanGTP dissociates karyopherin α from karyopherin β1 (77, 100),

presumably by forming the same intermediate ternary complex. However, karyopherin β1 preferentially binds to RanGTP over karyopherin α (77, 101). This difference in affinity would force the RanGTP/karyopherin β1 disassembly reaction in the direction of RanGTP/karyopherin β1 complex formation (Fig. 14, step 1). However, in the presence of C-Nup1, and either RanGAP and RanBP1 or RanGEF, the equilibrium is shifted towards formation of the karyopherin α/β1 complex (Fig. 6, 7 and 9*B*). The presence of a GST-NLS fusion protein did not affect the disassembly reaction when tested in the GTP hydrolysis or surface plasmon resonance experiments (Floer, M. and Blobel, G., unpublished observation).

GTP hydrolysis is not required for the release of karyopherin β1 from RanGTP by karyopherin α; this is evidenced by the fact that release of karyopherin β1 from RanGMP-PCP occurs with the same efficiency as release from RanGTP (Fig. 10*C*). This finding offers new insight into the function of small Ras-like GTP-binding proteins. GTP-binding proteins switch between an active GTP bound form and an inactive GDP bound form (reviewed in (102)). The GTP bound protein often forms a complex with a downstream effector molecule. This interaction is thought to be terminated by GTP hydrolysis (see for example

interaction of Ras with Raf-kinase (94)) as the GDP bound form generally has a lower affinity for the effector than the GTP bound form. As GTP-binding proteins are often resistant to GTPase activating proteins when bound to an effector (92), intrinsic GTP hydrolysis is thought to trigger complex disassembly (94). Our results on RanGTP/karyopherin β1 disassembly suggest that instead a "release factor" terminates the interaction between the GTP-binding protein and the effector. In our case karyopherin α is the release factor. Release factors analogous to karyopherin α may exist for other GTP-binding proteins. The karyopherin α dependent release of RanGTP from karyopherin β1 occurs much faster than intrinsic hydrolysis of Ran bound GTP ($k_{release} > 0.1\ s^{-1}$ compared to $k_{cat} = 5 \times 10^{-5}\ s^{-1}$ (95)). Our results also suggest that RanGTP/karyopherin β1 dissociation in the absence of karyopherin α does not require GTP hydrolysis, as dissociation of the RanGMP-PCP/karyopherin β1 complex occurred with the same apparent rate as the dissociation of the RanGTP/karyopherin β1 complex under the conditions described (Floer, M. and Blobel, G., data not shown)(Fig. 10).

RanGAP stimulates disassembly of the RanGTP/karyopherin β1 complex (Fig. 6) by converting the released RanGTP to RanGDP thereby preventing reformation of the complex. We found that RanGEF can replace RanGAP in the

disassembly reaction, as seen in the nucleotide exchange assay (Fig. 8*A*). In the solution binding assay, however, efficient disassembly of the RanGTP/karyopherin β1 complex can occur only in the presence of RanGEF and GDP but not GTP (Fig. 9*A*, lane 4). This result suggests that in the presence of GTP the released RanGTP is reloaded with new GTP instead of being converted to RanGDP. This presumably shifts the equilibrium to reformation of the RanGTP/karyopherin β1 complex during the 1 h reaction time (Fig. 9*A*, lane 5). Nevertheless, considering the mechanism of nucleotide exchange by RanGEF (33), one could propose that RanGEF binding to the released RanGTP results in dissociation of the nucleotide and formation of a nucleotide free Ran/RanGEF complex (Fig. 14, step 4). This complex could then be disassembled in the presence of Mg^{2+} and GTP or GDP. However, if RanGEF concentrations are high, this nucleotide free Ran/RanGEF complex might exist as a stable intermediate. In fact, isolation of RanGEF from HeLa cell nuclei revealed that a large fraction of nuclear RanGEF is in complex with Ran (103). This complex could be dissociated in the presence of Mg^{2+} and nucleotide, suggesting that the nuclear Ran/RanGEF complex is indeed nucleotide free (25). RanGAP, apart from being a cytosolic protein (38), is also localized at the NPC (39-41) and in the nucleoplasm (104). In

contrast, RanGEF is located mainly in the nucleoplasm bound to chromatin (34, 35, 103), although it may also bind to nucleoporins (Floer, M. and Blobel, G., unpublished observation)(Fig. 9*A*). However, both proteins have been shown to play a role in nuclear protein import (41, 71, 73). One could therefore speculate that disassembly of RanGTP/karyopherin β1 *in vivo* may occur in the presence of both RanGAP and RanGEF at the NPC. However, on the nuclear side of the NPC disassembly of RanGTP/karyopherin β1 complex is probably stimulated by RanGEF. The thereby formed Ran/RanGEF complex might be dissociated again in the presence of GTP. The newly generated RanGTP could then be used for export of proteins from the nucleus as will be discussed in chapter 4.

Our results suggest that RanBP1 has three functions. First, RanBP1 binds to RanGTP after its release from karyopherin β1 by karyopherin α and prevents reformation of the RanGTP/karyopherin β1 complex when C-Nup1 is present (Fig. 9*B*). Second, RanBP1 interaction with RanGTP increases the affinity of RanGTP for RanGAP which results in stimulation of GAP activity (Fig. 7 and 13)(75, 76, 86). Both these functions contribute to stimulating disassembly of the RanGTP/karyopherin β1 complex. Third, RanBP1 binding to RanGTP prevents interaction of RanGTP with RanGEF (Fig. 8*B*)(75, 86). Whether inhibition of

RanGEF by RanBP1 occurs in the cell and what role it plays in controlling Ran remains to be determined. RanBP1, RanGTP, and karyopherin β1 have been proposed to form a ternary complex (86, 105-107), which may assemble during release of RanGTP from karyopherin β1. However, in the presence of karyopherin α and C-Nup1 this complex is unstable as the majority of karyopherin β1 bound to C-Nup1 without associated RanGTP and RanBP1 (Fig. 9*B*). We propose that RanBP1 functions to promote recycling of RanGTP and karyopherin β1 at the NPC. Our proposal is consistent with the localization of RanBP1 in the cytoplasm and at the nuclear envelope (76) and its proposed involvement in protein import (76, 106).

The C-terminus of Nup1 binds karyopherin α/β1 and stimulates RanGTP/karyopherin β1 disassembly (Fig. 6*B*). We suggest that C-Nup1 sequesters karyopherin β1 and inhibits reformation of the RanGTP/karyopherin β1 complex (Fig. 11). We also suggest that NSP1, Nup2, Nup36 and the FXFG-peptide repeat region of Nup1 may function in a manner similar to C-Nup1 (Fig. 12*B*), since they also bind karyopherin α/β1 (46, 77). Nup36 was initially identified as a karyopherin α/β1 binding protein (46) and was later shown to bind RanGTP (Floer, M., Nehrbass, U. and Blobel, G., unpublished observation)(88).

Overexpression of a tagged version of Nup36 results in its localization to the nucleoplasm (88). However, Nup36 localizes to the nuclear envelope as visualized by immunofluorescence using antibodies against Nup36 (Nehrbass, U. and Blobel, G., personal communication). In addition to a karyopherin α/β1 binding site, Nup36 has a Ran-binding domain similar to the one in RanBP1 (89, 90). Nup36 stimulates GTP hydrolysis by RanGAP as does RanBP1 (Fig. 13)(88), and is synthetically lethal with RanGAP (88). This genetic interaction can be explained by our *in vitro* data as both Nup36 and RanGAP cooperate to recycle Ran and karyopherin β1 for another round of function. Other nucleoporins, like the mammalian Nup358 (108, 109) and yeast Nup2 (89, 90) also contain Ran-binding domains and may function in a manner similar to Nup36.

The involvement of different nucleoporins in the disassembly of the RanGTP/karyopherin β1 complex has interesting implications for protein transport into the nucleus. Nucleoporins that bind karyopherin α/β1 have different activities in the disassembly of the RanGTP/karyopherin β1 complex via karyopherin α (Fig. 12*B*). This may be due to different affinities of the nucleoporins for karyopherin α/β1. If nucleoporins are localized along the NPC with increasing affinities for karyopherin α/β1 (from cytoplasmic to nucleoplasmic sites),

disassembly of RanGTP/karyopherin β1 complexes and concomitant docking of karyopherin α/β1 might occur along an affinity gradient. This affinity gradient may confer directionality to movement of transport factors and substrates across the nuclear pore complex.

Our results, together with previous results by other investigators (77) did not identify a distinct step in the mechanism of nuclear protein import that does require GTP hydrolysis on Ran. However, we found that RanGAP stimulates disassembly of the RanGTP/karyopherin β1 complex in the presence of karyopherin α, indicating that recycling of RanGTP and karyopherin β1 is stimulated by RanGAP (Fig. 6). RanGAP thereby converts Ran to its GDP bound form, which might be required for another round of Ran function. This finding is in agreement with recent findings by Schwoebel et al. who found that import of a cNLS-protein into the nucleus of digitonin-permeabilized cells occurred in the presence of Ran and GMP-PNP or in the presence of the RanQ69L mutant, which cannot interact with RanGAP (27). However, the import rates seen in the presence of GMP-PNP or the RanQ69L mutant were lower than those seen in the presence of wild type Ran and GTP. This finding might suggest that single turnover import does not require GTP hydrolysis on Ran. However, if Ran is limiting in the

reaction, recycling of Ran and its conversion to the GDP bound form by RanGAP might be rate limiting. Interestingly, recent studies indicate that GTP hydrolysis on Ran might occur after the RanGTP/karyopherin β1 complex has docked on a RBH-domain of the nucleoporin Nup358 (Yaseen, N. and Blobel, G., manuscript in preparation). However, further studies are necessary to elucidate when GTP hydrolysis occurs *in vivo*.

Chapter 4: Mechanism of Crm1-mediated protein export from the nucleus

4.1 Introduction on nuclear protein export

Early studies in Xenopus oocytes revealed that a single NPC can mediate not only import but also export of particles (110, 111). Nuclear microinjection experiments with gold particles of different sizes that had been coated with tRNA, 5S RNA or poly(A) demonstrated that export occurred through the central channel of the NPC (110). Export of RNA coated gold particles was found to be a saturable process. The size limit for particles that were exported was 230 Å, and export required an RNA coat. Double injection experiments with RNA coated and nucleoplasmin coated gold particles further demonstrated that an individual NPC can mediate both import and export at the same time. Later experiments suggested that export of different RNAs might occur via different pathways, since export of tRNA, mRNA, U snRNA and ribosomal RNA were each saturable processes but did not necessarily saturate the export of one another (112-114). Recently, it was shown that U snRNA, 5S RNA and proteins that contain an NES (nuclear export signal) might share a common pathway (115).

NES-mediated protein export was the first export pathway for which the cognate karyopherin, Crm1 (also known as exportin1, Xpo1 or Kap124), was

identified (116-120). CRM1 was originally discovered in *S. cerevisiae* as a gene involved in chromosomal region maintenance (121). Recently, the human homolog was found to be a soluble protein that interacts with the nucleoporin Nup214/CAN (122). Evidence that Crm1 is a karyopherin came from inhibitor studies with the antifungal drug leptomycin B (LMB). In *Schizosaccharomyces pombe* LMB was shown to target Crm1 (123). When LMB was found to inhibit nuclear export of the HIV-1 protein Rev, Crm1 was suggested to be the karyopherin that exports Rev from the nucleus (116).

Rev has been shown to transport unspliced viral mRNA from the nucleus to the cytoplasm (124-126). An RNA-binding site in Rev was identified and shown to interact with a specific Rev response elements (RRE) in viral mRNA (127-129). Rev was further shown to contain an NES; mutational analysis of Rev indicated that a leucine-rich stretch of amino acids (LPPLERLTLD) is required and sufficient for export of Rev from the nucleus (115, 128, 130). Similar NESs were also found in other proteins (131-133), which suggested the existence of a receptor that recognizes an NES and exports the protein from the nucleus. Recent studies have shown direct binding of Crm1 to an NES-peptide (117-119). Some authors have found a requirement of RanGTP for Crm1/NES interaction, and proposed the

formation of a cooperative complex between Crm1, NES and RanGTP (117). UV-crosslinking and protein footprinting studies have recently indicated that Crm1 and NES interact in the absence of Ran (134). However, a ternary complex is formed when RanGTP is present. The interaction between Crm1 and NES was found to be sensitive to LMB (117-119). Interestingly, a more detailed biochemical analysis of these interactions revealed that only formation of the ternary Rev/Crm1/RanGTP complex is inhibited by LMB (134). Together these results explain the inhibitory effect of LMB on Rev export (116).

Rev has been studied extensively and several interactions of Rev with other proteins were established using yeast two-hybrid screens or a copper resistance assay. Among these proteins the Rev interacting protein Rip1 was identified (135, 136). Rip1 was shown to be a nucleoporin, and to contain FG-peptide repeats (136) and has therefore been termed Nup42. Using a copper resistance assay in *S. cerevisiae* that measures pre-mRNA export of Rev from the nucleus the Nup42/Rev interaction was found to be bridged by Crm1 (137). Furthermore, Crm1 was shown to export a reporter NES-protein in *S. cerevisiae* (120). Crm1 dependent export has been shown for a number of NES containing proteins among them Mdm2, which mediates nuclear export of the tumor suppressor gene product

p53 (138, 139), and cyclin B1, a protein required for the onset of mitosis (140-142). Together these findings defined a Crm1-mediated nuclear export pathway. However, the molecular mechanism of Crm1-mediated protein export is unknown.

4.2 Results

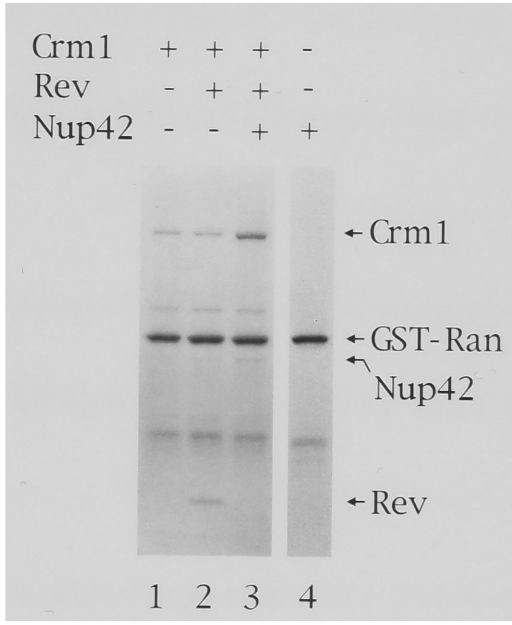
We used the recombinant HIV-1 protein Rev and various recombinant proteins from *S. cerevisiae* and employed a combination of solution binding assays and RanGTP hydrolysis and nucleotide exchange assays to detect reaction intermediates in the Crm1-mediated nuclear export of Rev.

4.2.1 Interaction of a Rev/Crm1/RanGTP complex with Nup42 results in release of Rev and formation of a Nup42/Crm1/RanGTP complex

A likely candidate for a Crm1 interacting nucleoporin is Nup42, based on previously reported *in vivo* data (135-137). To investigate complex formation between Crm1, Nup42, Rev and RanGTP we immobilized GST-Ran that was 80-90 % GTP bound (see Experimental Procedures) on glutathione-beads. The final concentration of GST-RanGTP in these experiments was 3 μM. We found that Rev and Crm1 form a ternary complex with RanGTP (Fig. 15, lane 2) as has been previously described (134). We did not detect an increase in the amount of Crm1 bound to GST-RanGTP in the presence of 1.5 μM Rev (lane 2) over the levels seen in the presence of Crm1 alone (lane 1). This result indicates that Rev does not increase the affinity of Crm1 for RanGTP, if present at a concentration of

Fig. 15 Rev and Nup42 binding to Crm1/RanGTP is mutually exclusive.

Solution binding assays were performed as described in the Experimental Procedures. GST-RanGTP was immobilized on glutathione-beads. Then 1.8 μM Crm1 (lanes 1-3), 1.5 μM Rev (lanes 2 and 3) and 0.375 μM Nup42 (lanes 3 and 4) was added. Reactions were incubated for 30 min at 21°C. The proteins bound to GST-RanGTP were analyzed by SDS-PAGE as described in the Experimental Procedures.


Crm1 + + + -
Rev - + + -
Nup42 - - + +
Crm1
GST-Ran
Nup42
Rev
1 2 3 4

1.5 μM. If Nup42 was added together with Rev and Crm1, a Nup42/Crm1/ RanGTP complex was formed (lane 3). In the presence of Nup42 the amount of Crm1 recruited to GST-RanGTP was significantly increased (compare lanes 1 and 3), suggesting that Crm1, RanGTP and Nup42 might form a cooperative complex. However, Rev was excluded from this complex (lane 3). Nup42 did not bind to GST-RanGTP in the absence of Crm1 (lane 4). Together these results suggest that Rev is released, when the Rev/Crm1/RanGTP complex binds to Nup42.

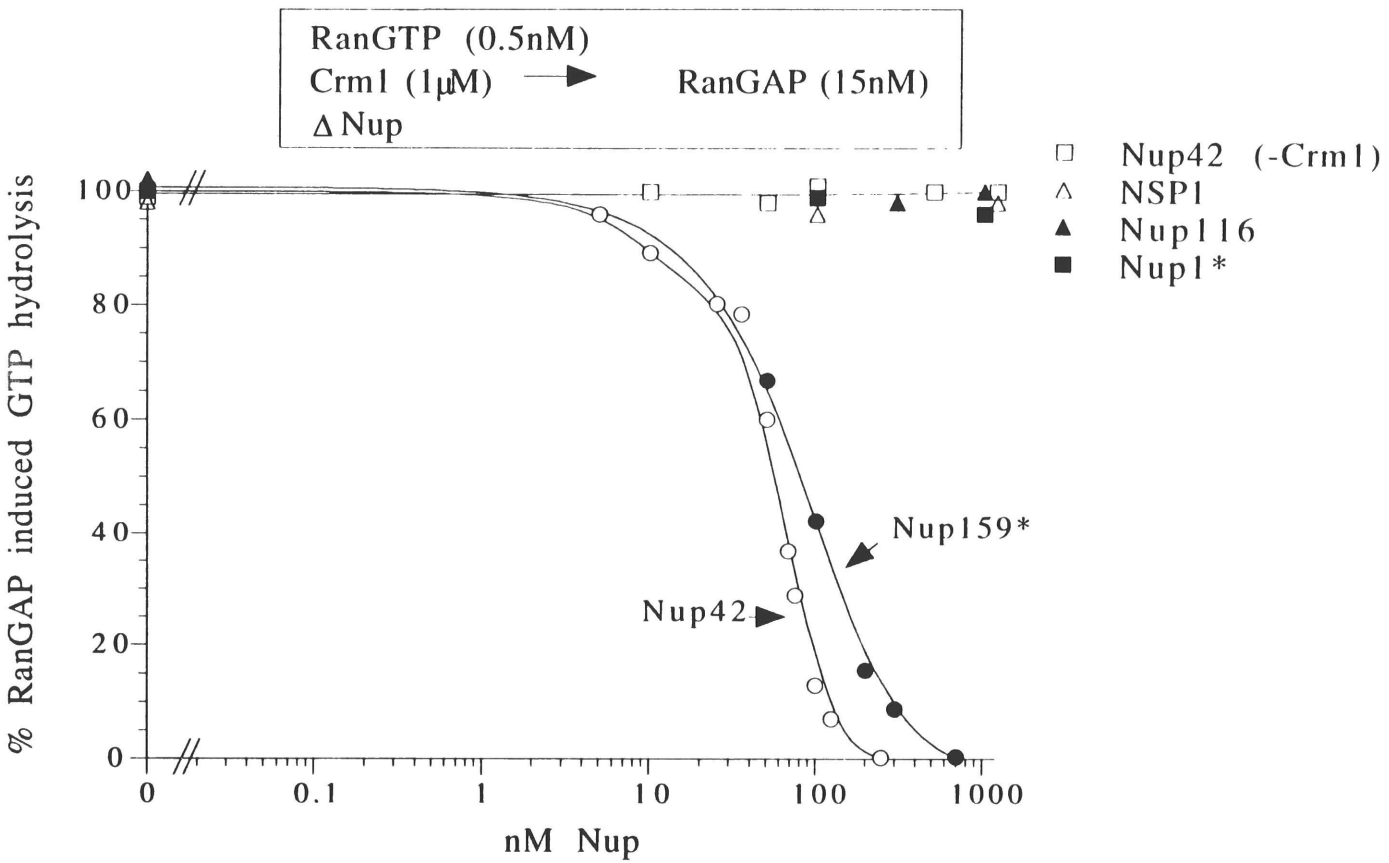
4.2.2 Crm1 and RanGTP form complexes with distinct nucleoporins that are protected against GTP hydrolysis by RanGAP and GTP exchange by RanGEF

We have previously used protection of RanGTP from RanGAP induced GTP hydrolysis to measure RanGTP complex formation (82). We therefore investigated whether the Nup42/Crm1/RanGTP complex is protected against RanGAP (Fig. 16). Preincubation of 0.5 nM RanGTP with increasing amounts of Nup42 in the presence, but not in the absence of 1 μM Crm1 (compare Fig. 16, open circles to open squares) resulted in complete inhibition of RanGAP stimulated GTP hydrolysis. Incubation of RanGTP with 1 μM Crm1 alone did not

Fig. 16 Crm1 and RanGTP form a ternary complex with the nucleoporins Nup42 and Nup159 respectively that is protected against RanGAP.

GTP hydrolysis assays were performed as described in the Experimental Procedures. 0.5 nM Ran-[γ-^{32}P]GTP was incubated for 15 min at 21°C with (open circles, closed circles, open triangles, closed triangles, closed squares) or without (open squares) 1 μM Crm1 and increasing amounts of Nup42 (open circles, open squares), Nup159* (closed circles), NSP1 (open triangles), Nup116 (closed triangles) or Nup1* (closed squares). Then 15 nM RanGAP was added, and the reactions were incubated for 20 min at 21°C. The extent of GTP hydrolysis was quantified as described in the Experimental Procedures.

Nup159* signifies a fragment containing the FG-peptide repeat region of Nup159, Nup1* signifies a fragment containing the FXFG-peptide repeat region of Nup1.

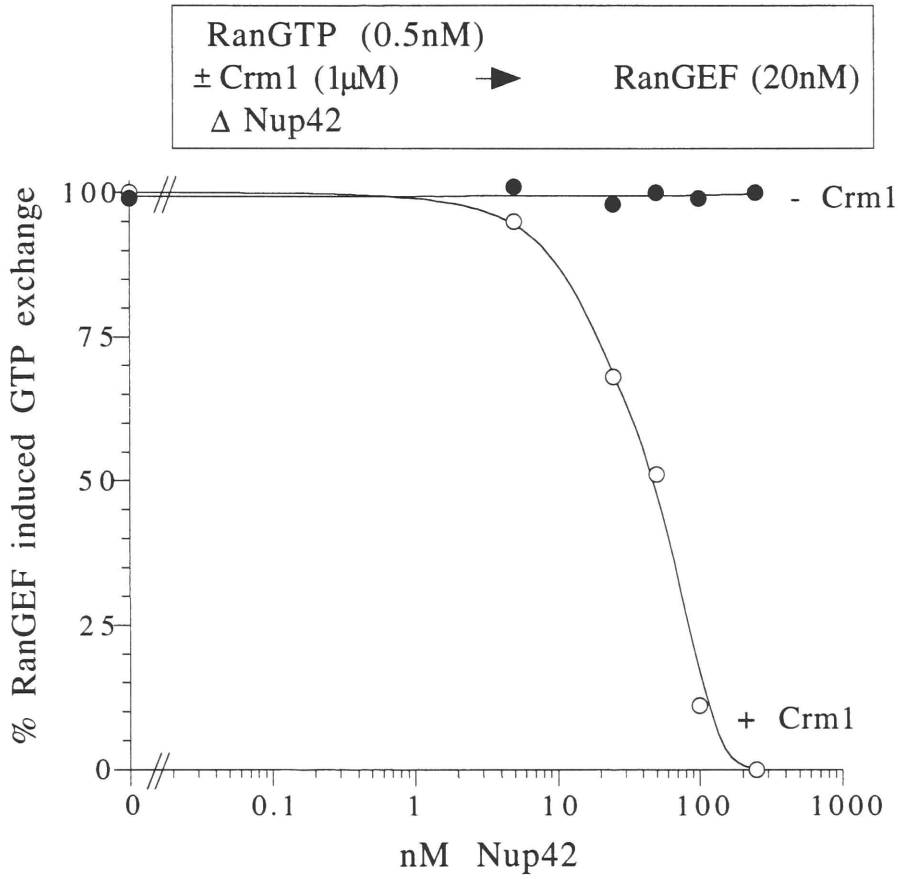

RanGTP (0.5nM)
Crm1 (1μM)
ΔNup
RanGAP (15nM)
Nup42 (-Crm1)
NSP1
Nup116
Nup1*
% RanGAP induced GTP hydrolysis
100
80
60
40
20
0
0
0.1
1
10
100
1000
nM Nup
Nup159*
Nup42

result in detectable levels of RanGAP inhibition (not shown). Together, the results from Fig. 15 and Fig. 16 suggest that Crm1, RanGTP and Nup42 form a cooperative complex, with Nup42 increasing the affinity of Crm1 for RanGTP. A constant for RanGTP dissociation from the Nup42/Crm1/RanGTP complex is estimated to be 60 nM. Crm1 and RanGTP were also found to form a ternary complex with Nup159*, a fragment of the nucleoporin Nup159 containing its FG-peptide repeat region (65)(Fig. 16, closed circles; for the Nup159 construct see Fig. 12*A*). However, the affinity of this complex for RanGTP was lower and a K_D of 100 nM was estimated. These estimates reflect average dissociation constants, since the stoichiometry of the Nup42/Crm1/RanGTP or Nup159*/Crm1/RanGTP complex is not known. Crm1 and RanGTP did not form a complex with the nucleoporins NSP1, Nup116 or Nup1*, a fragment of Nup1 containing its FXFG-peptide repeat region (for the Nup constructs see Fig. 12*A*). Addition of 1 μM NSP1 (open triangles), 1 μM Nup116 (closed triangles) or 1 μM Nup1* (closed squares) in the presence of 1 μM Crm1 did not result in inhibition of RanGAP stimulated GTP hydrolysis.

Complex formation of Crm1, RanGTP and Nup42 was also found to inhibit GTP exchange by RanGEF (Fig. 17). In the presence of 1 μM Crm1 and

Fig. 17 The Nup42/Crm1/RanGTP complex is protected against RanGEF induced GTP exchange.

GTP exchange assays were done as described in the Experimental Procedures. 0.5 nM Ran-[α-^{32}P]GTP was incubated with (open circles) or without (closed circles) 1 μM Crm1 and Nup42 at the concentrations indicated for 15 min at 21°C. Then 20 nM RanGEF was added, and the reactions were incubated for 20 min at 21°C. The extent of GTP exchange was quantified as described in the Experimental Procedures.


RanGTP (0.5nM)
± Crm1 (1μM)
Δ Nup42
RanGEF (20nM)
% RanGEF induced GTP exchange
100
75
50
25
0
- Crm1
+ Crm1
0
0.1
1
10
100
1000
nM Nup42

increasing amounts of Nup42 GTP exchange on Ran by 20 nM RanGEF was inhibited (Fig. 17, open circles). Addition of 1 μM Rev together with 1 μM Crm1 did not inhibit RanGAP induced GTP hydrolysis or GTP exchange by RanGEF (not shown). Neither did addition of 1 μM Rev together with 1 μM Crm1 and increasing amounts of Nup42 further stimulate the inhibition seen in the presence of Crm1 and Nup42 (not shown). Inhibition of RanGEF induced exchange of GTP was also seen in the presence of Crm1 and Nup159* (not shown). Together these results indicate that Crm1, RanGTP and Nup42 or the FG-peptide repeat containing fragment of Nup159 form a complex independently of an NES-containing export substrate.

4.2.3 Release of docked Crm1/RanGTP from Nup42 by RanBP1, and formation of a ternary Crm1/RanGTP/RanBP1 complex

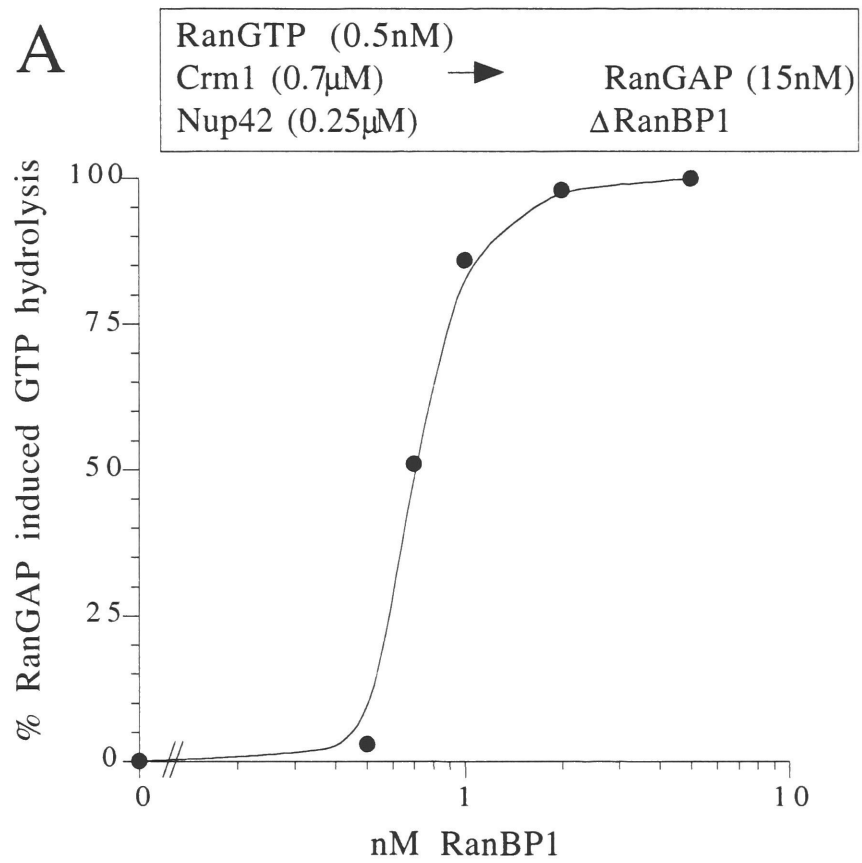
How are Crm1 and RanGTP released from Nup42? Since complex formation of RanGTP with Crm1 and Nup42 results in inhibition of RanGAP induced GTP hydrolysis, we assayed for complex disassembly by measuring release of RanGAP inhibition. Addition of the Ran binding protein RanBP1 to Nup42/Crm1/RanGTP complex completely restored RanGAP induced GTP

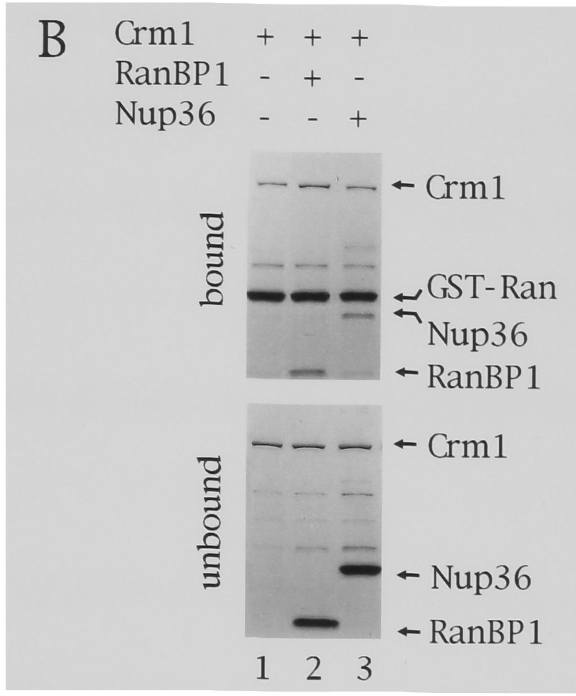
hydrolysis (Fig. 18*A*). For these experiments 0.5 nM RanGTP was preincubated with 0.7 μM Crm1 and 0.25 μM Nup42. Addition of increasing amounts of RanBP1 in the presence of 15 nM RanGAP completely restored RanGAP induced GTP hydrolysis. 50 % RanGAP induced GTP hydrolysis occurred in the presence of 0.7 nM RanBP1. The concentration of RanGTP used in this experiment is too high to allow an accurate measurement of the interaction between RanBP1 and Nup42/Crm1/RanGTP complex. However, an estimate for the free concentration of RanBP1 at 50 % GTP hydrolysis of 0.45 nM is close to the K_D for the RanBP1/RanGTP interaction, which has been determined to be between 0.1 nM and 0.6 nM for mammalian proteins (72, 143, 144).

To further understand the mechanism of Crm1/RanGTP release from Nup42 by RanBP1, we investigated whether Crm1, RanGTP and RanBP1 form a complex. For these experiments GST-RanGTP was immobilized (Fig. 18*B*). Addition of RanBP1 in the presence of Crm1 resulted in binding of RanBP1 to RanGTP and an increase in Crm1 binding to RanGTP compared to Crm1 alone (Fig. 18*B*, compare lanes 1 and 2). This result indicates the formation of a cooperative complex between Crm1, RanGTP and RanBP1. Together these results indicate that RanBP1 dissociates the Crm1/RanGTP complex from Nup42,

Fig. 18 RanBP1 releases Crm1/RanGTP from Nup42, thereby forming a ternary Crm1/RanGTP/RanBP1 complex.

A, GTP hydrolysis assays were performed as described in the Experimental Procedures. 0.5 nM Ran-[γ-^{32}P]GTP was incubated with 0.7 μM Crm1 and 0.25 μM Nup42 for 15 min at 21°C. Then 15 nM RanGAP and increasing amounts of RanBP1 were added, and the reactions were incubated for 20 min at 21°C. The extent of GTP hydrolysis was quantified as described in the Experimental Procedures. *B*, Solution binding assays were done as described in the Experimental Procedures. Immobilized GST-RanGTP was incubated with 0.25 μM Crm1 (lanes 1-3), 1.7 μM RanBP1 (lane 2) or 0.8 μM Nup36 (lane 3) for 30 min at 21°C. The bound and unbound fractions were analyzed by SDS-PAGE as described in the Experimental Procedures.


A
RanGTP (0.5nM)
Crm1 (0.7μM)
Nup42 (0.25μM)
RanGAP (15nM)
ΔRanBP1
% RanGAP induced GTP hydrolysis
100
75
50
25
0
0
1
10
nM RanBP1


B
Crm1 + + +
RanBP1 - + -
Nup36 - - +
bound
unbound
Crm1
GST-Ran
Nup36
RanBP1
Crm1
Nup36
RanBP1
1 2 3

thereby forming a ternary Crm1/RanGTP/RanBP1 complex.

Interestingly, we found that Nup36, also known as Yrb2, can disassemble Crm1/RanGTP from Nup42 (not shown). Nup36 is one of two RBH (RanBP1-homologous) domain containing proteins in *S. cerevisiae*, the other being Nup2 (46, 88-90). We have also found that Nup36 forms a ternary complex with RanGTP and Crm1 in the GST-binding assay (Fig. 18*B*, lane 3). These data indicate that Nup36 and RanBP1 might have overlapping functions. However, the exact function of a Crm1/RanGTP/Nup36 complex remains to be determined.

4.2.4 RanGEF-mediated disassembly of the Crm1/RanGTP/RanBP1 complex

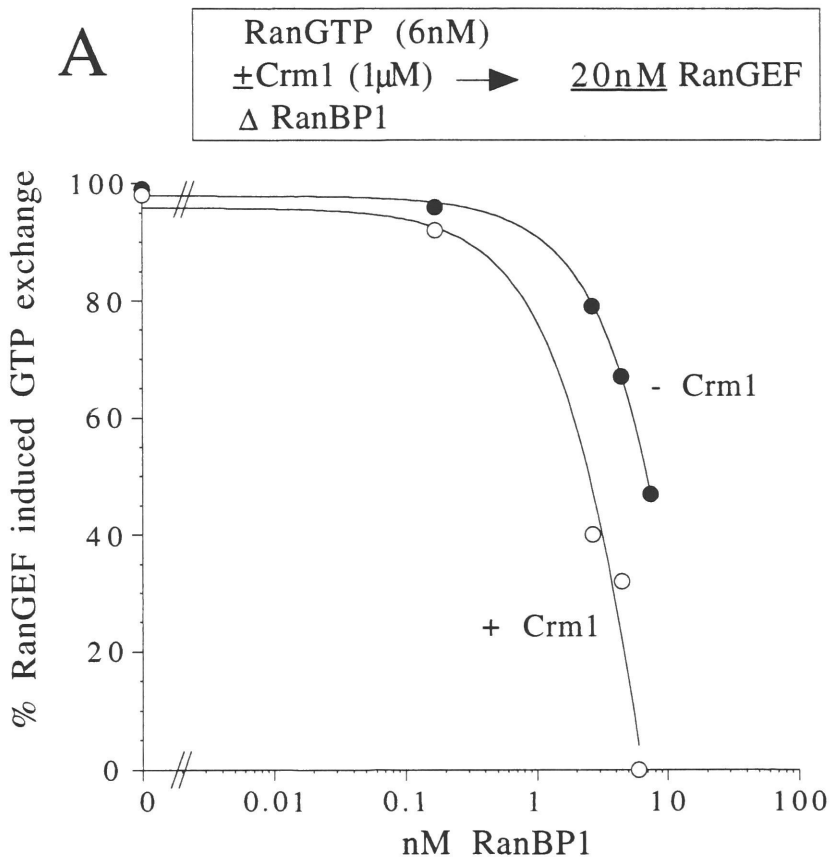
The ternary Crm1/RanGTP/RanBP1 complex that is formed, when Crm1 and RanGTP are released from Nup42, is susceptible to RanGAP induced GTP hydrolysis (Fig. 18*A*). GTP hydrolysis of Ran bound GTP is likely to disassemble the Crm1/RanGTP/RanBP1 complex. Surprisingly, we found an alternative mechanism of Crm1/RanGTP/RanBP1 complex disassembly by the exchange factor RanGEF. Preincubation of about 6 nM RanGTP with 1 μM Crm1 and increasing amounts of RanBP1 inhibited GTP exchange by 20 nM RanGEF (Fig.

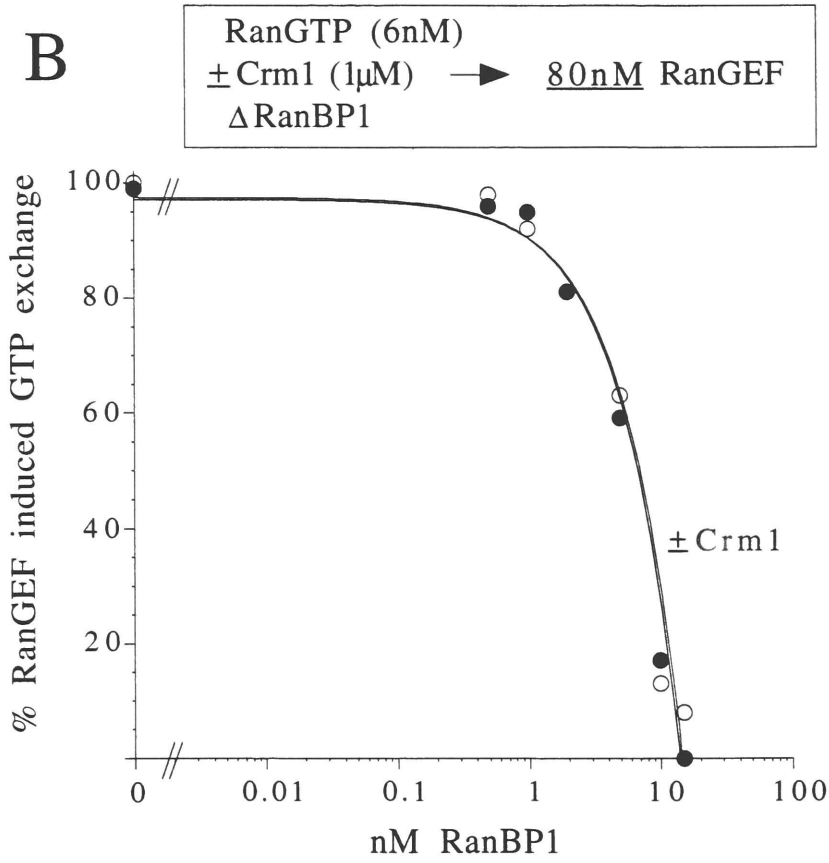
19*A*, open circles). Surprisingly, if the concentration of RanGEF was increased to 80 nM, the Crm1/RanGTP/RanBP1 complex was no longer protected against RanGEF induced GTP exchange (Fig. 19*B*, open circles). Since the concentrations of Crm1 and RanBP1 were sufficient to allow for Crm1/RanGTP/RanBP1 complex formation under these conditions (compare with Fig. 19*A*, open circles), this result indicates that the Crm1/RanGTP/RanBP1 complex that is formed when all three components are preincubated, is disrupted by 80 nM RanGEF. Interestingly, the RanGTP/RanBP1 complex that is formed at higher concentrations of RanBP1 was not sensitive to increased concentrations of RanGEF (compare Fig. 19*A*, closed circles to Fig. 19*B*, closed circles). These data suggest that the Crm1/RanGTP/RanBP1 ternary complex can be attacked by RanGEF if present at sufficiently high levels. However, since in this assay we only measure release of GTP, we could not determine what happens to the complex after GTP has been released. Most likely however, release of GTP weakens the affinity of Crm1 and RanBP1 for RanGTP, resulting in disassembly of the complex and formation of an intermediate Ran/RanGEF complex.

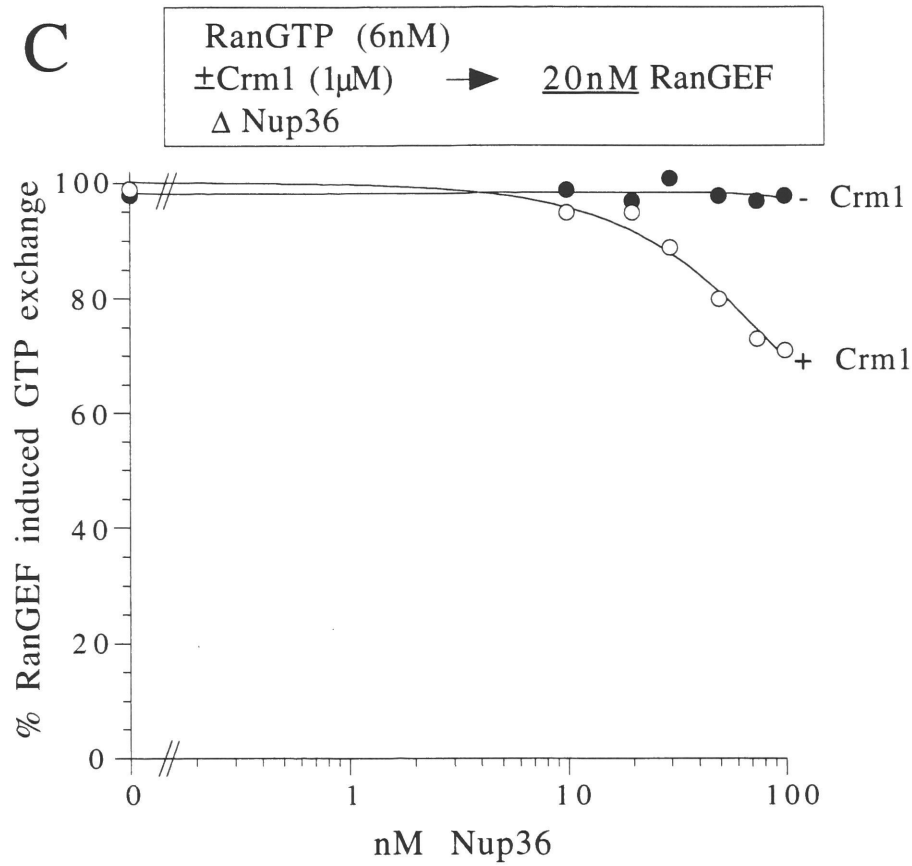
We found that the Crm1/RanGTP/Nup36 complex is also sensitive to increased concentrations of RanGEF. In the presence of 1 μM Crm1 and increasing

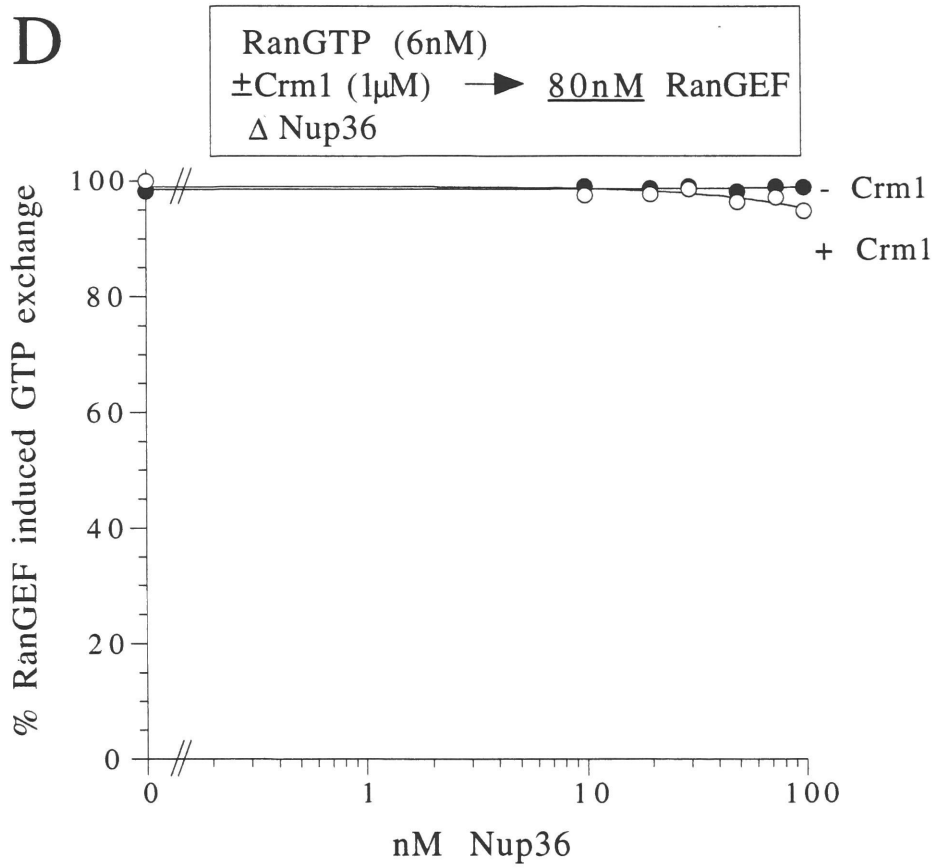
Fig. 19 The Crm1/RanGTP/RanBP1 complex is sensitive to increased concentrations of RanGEF.

GTP exchange assays were done as described in the Experimental Procedures. *A* and *B*, 6 nM Ran-[α-^{32}P]GTP was incubated with (open circles) or without (closed circles) 1 μM Crm1 and increasing amounts of RanBP1 for 15 min at 21°C. Then 20 nM (*A*) or 80 nM (*B*) RanGEF was added, and the reactions were incubated for 20 min at 21°C. *C* and *D*, 6 nM Ran-[α-^{32}P]GTP was incubated with (open circles) or without (closed circles) 1 μM Crm1 and increasing amounts of Nup36 for 15 min at 21°C. Then 20 nM (*C*) or 80 nM (*D*) RanGEF was added, and the reactions were incubated for 20 min at 21°C.


A
RanGTP (6nM)
±Crm1 (1μM) → 20nM RanGEF
Δ RanBP1
% RanGEF induced GTP exchange
100
80
60
40
20
0
- Crm1
+ Crm1
0
0.01
0.1
1
10
100
nM RanBP1


B
RanGTP (6nM)
± Crm1 (1μM) → 80nM RanGEF
ΔRanBP1
% RanGEF induced GTP exchange
100
80
60
40
20
0
0.01
0.1
1
10
100
nM RanBP1
± Crm1


C
RanGTP (6nM)
±Crm1 (1μM)
Δ Nup36
20nM RanGEF
% RanGEF induced GTP exchange
100
80
60
40
20
0
0
1
10
100
nM Nup36
- Crm1
+ Crm1


D
RanGTP (6nM)
±Crm1 (1μM) → 80nM RanGEF
Δ Nup36
% RanGEF induced GTP exchange
100
80
60
40
20
0
0
1
10
100
nM Nup36
- Crm1
+ Crm1

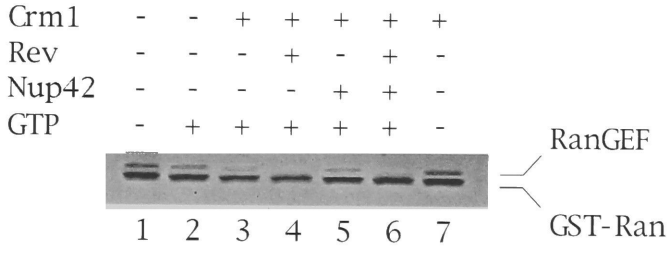
amounts of Nup36 GTP exchange on Ran by 20 nM RanGEF was inhibited (Fig. 19*C*, open circles). However, in the presence of 80 nM RanGEF the Crm1/RanGTP/Nup36 complex was attacked by RanGEF (Fig. 19*D*, open circles). We did not find complex formation between RanGTP and Nup36 in the absence of Crm1 at the concentrations of Nup36 used in these experiments (Fig. 19*C* and *D*, closed circles).

4.2.5 Crm1 and Rev disassemble Ran/RanGEF complex in the presence of GTP and Mg^{2+}

Nucleotide exchange by RanGEF is thought to occur through release of the nucleotide from Ran upon binding to RanGEF (33). This results in the formation of an intermediate nucleotide free Ran/RanGEF complex. Is dissociation of this intermediate complex and rebinding of GTP to Ran in the presence of Mg^{2+} stimulated by other factors? For these experiments GST-Ran was immobilized and preincubated with RanGEF. This resulted in the formation of a Ran/RanGEF complex (Fig. 20, lane 1). Addition of GTP in the presence of 2 mM $Mg(OAc)_2$ stimulated release of RanGEF from Ran as expected (lane 2). Interestingly, addition of Crm1 in the presence of GTP further stimulated release of RanGEF

Fig. 20 RanGEF is released from Ran in the presence of Mg^{2+}, GTP, Crm1 and Rev.

GST-Ran was immobilized as described in the Experimental Procedures, and then incubated with 1.7 μM RanGEF for 20 min at 21°C. After washing, 10 mM GTP (lanes 2-6), 2.2 μM Crm1 (lanes 3-7), 1.25 μM Rev (lanes 4 and 6) or 0.5 μM Nup42 (lanes 5 and 6) were added in the presence of 2 mM $Mg(OAc)_2$. The reactions were incubated for 10 min at 21°C. The fraction of RanGEF bound to GST-Ran was analyzed by SDS-PAGE as described in the Experimental Procedures.


Crm1 - - + + + + +
Rev - - - + - + -
Nup42 - - - - + + -
GTP - + + + + + -
RanGEF
GST-Ran
1 2 3 4 5 6 7

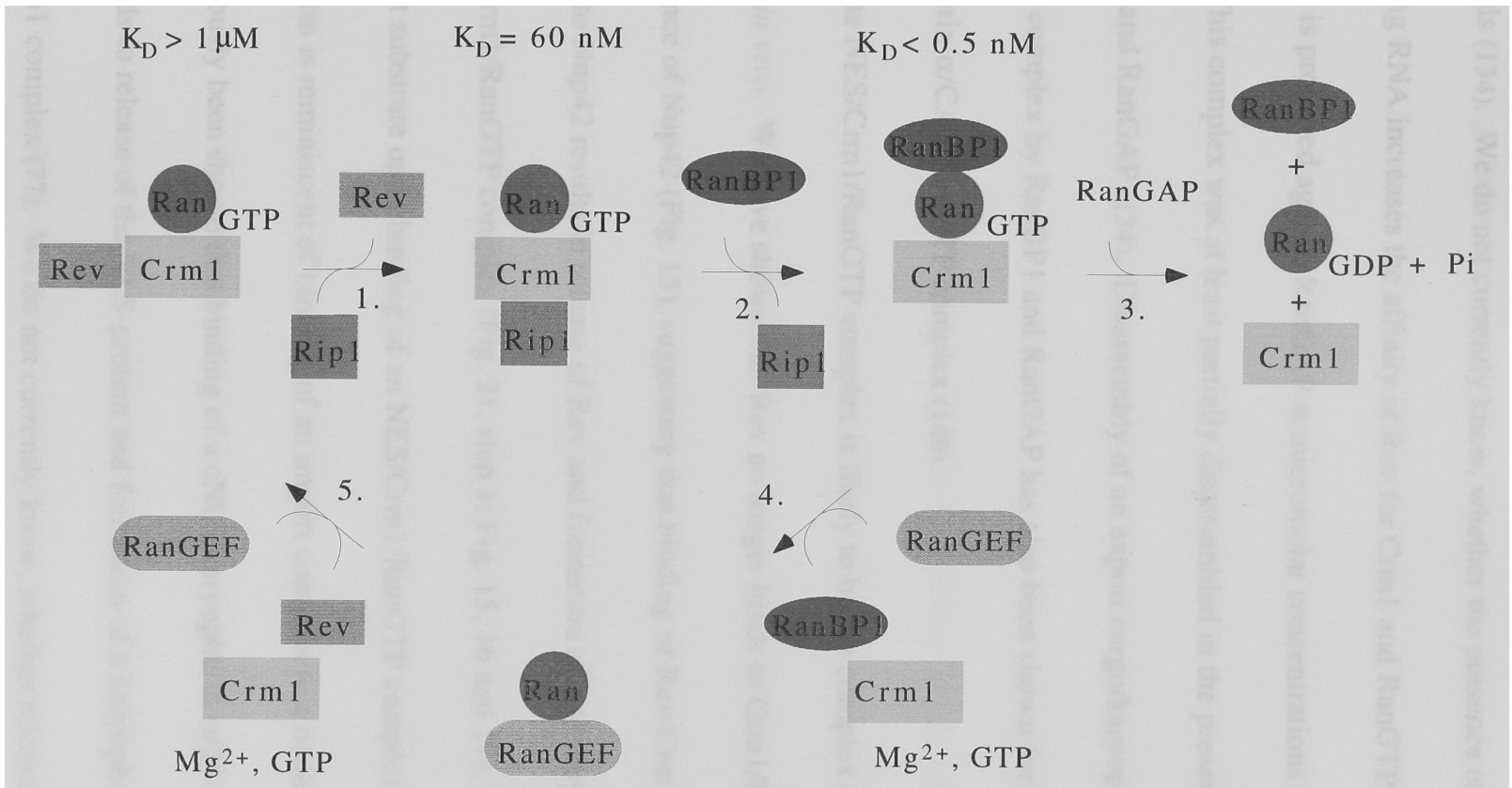
from Ran (lane 3). If Rev was added together with Crm1 all the bound RanGEF was released (lane 4). However, addition of Nup42 together with Crm1 did not stimulate release of RanGEF over the levels seen with Crm1 alone (lane 5). If Rev was added together with Nup42 and Crm1, release of RanGEF was restored (lane 6). These data suggest that binding of GTP and release of RanGEF from Ran is stimulated by Crm1 and Rev.

4.3 Discussion

We have analyzed the interactions between proteins involved in Crm1-mediated nuclear protein export. A model for the mechanism of nuclear protein export describing the different steps of the reaction is shown in Fig. 21. Export is presumably initiated by the formation of an NES-protein/Crm1/RanGTP complex in the nucleus (see Fig. 21; Fig. 15, lane 2), where RanGTP concentrations are thought to be high due to the predominantly nuclear localization of RanGEF (34, 35) and the low concentrations of RanGAP in the nucleus (38, 104). It has recently been described that a ternary Rev/Crm1/RanGTP complex is formed (134). This complex is also formed when Rev is bound to a RRE containing RNA (134, 145). Similar export complexes involving the substrate, the export karyopherin and RanGTP have been described for the export of karyopherin α/importin α via CAS/Kap109 (146), the human homolog of yeast Cse1 (147, 148), as well as t-RNA export via exportin-t (149), the human homolog of yeast Los1(150, 151). The Rev/Crm1/RanGTP complex has a low affinity for RanGTP, since RanGAP induced GTP hydrolysis on Ran is not inhibited in the presence of 1 μM Crm1 and 1 μM Rev (not shown). Recently, it has been reported that a RRE/Rev/Crm1/RanGTP complex is protected against RanGAP induced GTP

Fig. 21 Model for the mechanism of nuclear protein export.

Docking of the Rev/Crm1/RanGTP complex at Nup42 results in formation of a ternary Nup42/Crm1/RanGTP complex and release of Rev. Crm1 and RanGTP are released from Nup42 by RanBP1, whereby a ternary Crm1/RanGTP/RanBP1 complex is formed. The Crm1/RanGTP/RanBP1 complex can be disassembled by RanGAP induced GTP hydrolysis. Alternatively, the Crm1/RanGTP/RanBP1 complex can be disassembled by RanGEF induced release of GTP. A likely resulting binary Ran/RanGEF complex can be dissociated in the presence of Mg^{2+} and GTP. Dissociation of RanGEF from Ran is stimulated by Crm1 and Rev, which leads to the formation of a next Rev/Crm1/RanGTP complex. The K_D values indicated above the complexes are estimates for the dissociation constants for RanGTP from these complexes.


K_D > 1 µM
K_D = 60 nM
K_D < 0.5 nM
Ran GTP
Rev
Crm1
1.
Rip1
Ran GTP
Crm1
Rip1
RanBP1
2.
RanBP1
Ran GTP
Crm1
RanGAP
3.
RanBP1
+
Ran GDP + Pi
+
Crm1
5.
RanGEF
Rev
Crm1
Mg^{2+}, GTP
Ran
RanGEF
4.
RanGEF
RanBP1
Crm1
Mg^{2+}, GTP

hydrolysis (134). We do not currently know, whether the presence of a RRE containing RNA increases the affinity of Rev for Crm1 and RanGTP, so that RanGTP is protected against RanGAP at micromolar concentrations of Rev and Crm1. This complex was at least partially disassembled in the presence of RanBP1 and RanGAP (134). Disassembly of an export cargo/karyopherin/ RanGTP complex by RanBP1 and RanGAP has also been shown for the karyopherin α/CAS/RanGTP complex (146).

The NES/Crm1/RanGTP complex is likely to be the complex that docks at the NPC *in vivo*. We have shown that Rev no longer binds to Crm1/RanGTP in the presence of Nup42 (Fig. 15), suggesting that binding of Rev/Crm1/RanGTP complex to Nup42 results in release of Rev and formation of a ternary Nup42/Crm1/RanGTP complex (Fig. 21, step 1; Fig. 15, 16 and 17). Release of the export substrate upon binding of an NES/Crm1/RanGTP complex to a nucleoporin is reminiscent of binding of an import complex to a nucleoporin. It has previously been shown that binding of a cNLS/karyopherin α/β1 complex to Nup1 leads to release of the NLS-protein and formation of a karyopherin α/β1/Nup1 complex (77). We do not currently know, whether release of the substrate, i.e. the cNLS or NES-protein, upon docking at the NPC occurs *in vivo*.

However, one might speculate that release of the substrate upon docking is favorable, if the rate-limiting step in transport is diffusion of the substrate across the NPC. Dissociation of the substrate upon docking would immediately allow its diffusion independently of the release of its karyopherin.

Nup42 was originally identified as a Rev interacting protein in a yeast two-hybrid screen or using a copper resistance assay (135, 136). The interaction was later shown to be mediated by Crm1, since the Nup42/Rev interaction is impaired in cells expressing a mutant Crm1 protein (137). Our data indicate that Rev is released when Rev/Crm1/RanGTP binds to Nup42. This finding suggests that the Rev/Nup42 interaction seen in the yeast two-hybrid assay might constitute an intermediate complex between Rev, Crm1, RanGTP and Nup42. This quarternary complex would be short-lived under physiological conditions, but might be stabilized under the conditions of the two-hybrid assay. Alternatively, another factor might stabilize a Rev/Crm1/RanGTP/Nup42 complex *in vivo*. Nup42 has been shown to be a FG-peptide repeat containing nucleoporin (136). However, the exact localization of Nup42 at the NPC remains elusive. Involvement of Nup42 in export of Rev from the nucleus *in vivo* was suggested by microinjection experiments in *Xenopus* oocytes (152). Injection of a fragment of Nup42

containing its FG-peptide repeat region into the nucleus of *Xenopus* oocytes blocked export of Rev as well as export of some U snRNAs from the nucleus. This finding suggests that an export complex containing Rev is able to bind to Nup42 *in vivo*. However, since in this experiment Nup42 was mislocalized to the nucleus, its interaction with a Rev export complex inhibited export of Rev. Nevertheless, the gene encoding for Nup42 is not essential and a Nup42 deletion in yeast does not result in mislocalization of an NES-GFP-NLS reporter protein (120). This finding suggests that other nups might take over the function of Nup42 in its absence. Interestingly, Nup42 is highly homologous to the yeast nucleoporin Nup159 (136). Nup159 contains FG-peptide repeats, and has been localized to the cytoplasmic fibers of the NPC (65). We found that Crm1 and RanGTP form a ternary complex with the FG-peptide repeat region of Nup159 (Fig. 16). If multiple docking steps occur during transport of the NES-protein across the NPC, one could speculate that Nup159 constitutes one of the last docking sites at the cytoplasmic side of the NPC. Interestingly, injection of a fragment of Nup159 containing its FG-peptide repeat region into the nucleus of *Xenopus* oocytes also blocked Rev and U snRNA export from the nucleus (152) suggesting that Rev might dock at Nup159, when it is exported from the nucleus. Furthermore, Crm1

has been found in a complex with the mammalian nucleoporin Nup214/CAN (122). Nup214/CAN contains FG-peptide repeats and is the closest mammalian homolog of yeast Nup42 and Nup159 (136). We did not find complex formation of Crm1 and RanGTP with the nucleoporins NSP1, Nup116 or the FXFG-peptide repeat containing region of Nup1, when the nucleoporins were present at 1 μM concentrations in the GTP hydrolysis assay (Fig. 16). Neither was complex formation with these nucleoporins found in the presence of 1 μM Crm1 and 1 μM Rev (not shown). Interestingly, previous studies on Rev interactions with different nucleoporins using a copper resistance assay identified interactions of Rev with Nup42, the FG-repeat region of Nup159 and the GLFG-peptide repeat region of Nup100, respectively (136). However, the FXFG-peptide repeat regions of NSP1 or Nup1, or the GLFG-peptide repeat region of Nup116 did not interact with Rev in these assays. These results are in agreement with our data on complex formation *in vitro*. It might be possible that interaction of Rev bound Crm1 with these nucleoporins requires higher nucleoporin concentrations than the concentrations we have used in our GTP hydrolysis assays. However, our data suggest that only a subset of nucleoporins interact with the export karyopherin Crm1. Interestingly, binding of Crm1 to a peptide repeat containing nucleoporin

occurs in the presence of RanGTP (Fig. 16 and 3). This is in contrast to protein import, where RanGTP prevents binding of karyopherin β1 to some peptide repeat containing nucleoporins (77, 153) and releases a karyopherin α/β1 complex from the nup (77). However, recent findings indicate that binding of karyopherin α/β1 to a nucleoporin can also occur in the presence of RanGTP, if the nucleoporin contains a RBH domain like the mammalian Nup358 (154)(Yaseen, N. and Blobel, G., paper submitted for publication).

We show here that Crm1/RanGTP is released from Nup42 by the Ran interacting protein RanBP1 (Fig. 21, step 2; Fig. 18*A*). Release by RanBP1 results in formation of a ternary Crm1/RanGTP/RanBP1 complex (Fig. 18*B*, lane 2, and Fig. 19*A*). RanBP1 has been postulated to contain an NES (132). This might have interesting implications for the mechanism by which RanBP1 releases Crm1/RanGTP from Nup42. However, since the Crm1/RanGTP/RanBP1 complex has a much higher affinity for RanGTP than the Rev/Crm1/RanGTP complex as judged by their resistance to RanGEF induced GTP exchange (Floer, M. and Blobel, G., unpublished observation)(Fig. 18*A*), it is unlikely that RanBP1 interacts with Crm1 and RanGTP only through its NES. The Crm1/RanGTP/RanBP1 complex is susceptible to RanGAP stimulated GTP

hydrolysis (Fig. 21, step 3; Fig. 18*A*). GTP hydrolysis is likely to disassemble the complex. Disassembly of the Crm1/RanGTP/RanBP1 complex via RanGAP might occur at the cytoplasmic side of the NPC, where RanGAP concentrations are thought to be high (38). However, RanGAP stimulated GTP hydrolysis on Ran is not required for single turnover export, as previous data from microinjection experiments have suggested (155). Injection of the RanG19V mutant, which cannot interact with RanGAP, into nuclei of cells did not affect export of an NES-reporter protein. This result indicated that transport across the NPC does not require RanGAP stimulated GTP hydrolysis on Ran. We have discovered an alternative way to disassemble the Crm1/RanGTP/RanBP1 complex that does not require RanGAP but the exchange factor RanGEF (Fig. 21, step 4; Fig. 19). Crm1/RanGTP/RanBP1 complex was found to be susceptible to increased concentrations of RanGEF, resulting in the release of GTP (Fig. 19*B*). Release of GTP might then weaken the interaction of Ran with Crm1 and RanBP1 and disassemble the complex. RanGEF might thereby form a binary complex with nucleotide-free Ran. It was also shown previously that Ran, RanBP1 and RanGEF form a complex that is nucleotide free (75). Therefore, it might also be possible that an intermediate RanBP1/Ran/RanGEF complex is formed, when RanGEF

interacts with Crm1/RanGTP/RanBP1 complex. Our data on release of GTP from Crm1/RanGTP/RanBP1 complex by RanGEF were surprising, since a RanGTP/RanBP1 complex was found to be resistant to RanGEF at the concentrations tested (Fig. 19*B*). The concentrations of RanGEF required for disassembly of Crm1/RanGTP/RanBP1 complex might well be present on the nuclear side of the NPC (34, 103), or even inside the NPC itself. Our data are in agreement with a requirement for RanGEF in export of U snRNA (156). U snRNA export has been shown to occur via Crm1 (117) and competes with Rev export (115). Our data also shed new light on the observation that microinjection of the RanT24N mutant into nuclei of cells impairs export of an NES-reporter protein (155) or export of U snRNA (42). The RanT24N mutant has a decreased affinity for nucleotide and an increased affinity for RanGEF (33, 157). If RanT24N merely blocked export, because it would not allow formation of an NES/Crm1/RanGTP complex due to its decreased affinity for GTP, coinjection of RanGTP together with RanT24N should restore export. However, when RanG19V was injected together with RanT24N, export was not restored (155). This might indicate that the RanT24N mutant sequesters RanGEF. If RanGEF were necessary to disassemble the Crm1/RanGTP/RanBP1 complex, depletion of

RanGEF from the system by RanT24N would diminish disassembly of the Crm1/RanGTP/RanBP1 complex. This would prevent recycling of Crm1 and RanGTP for a new round of docking.

Nup36 forms a ternary complex with Crm1 and RanGTP (Fig. 18*B* and Fig. 19*C*), that is disassembled by high concentrations of RanGEF (Fig. 19*D*). Nup36 was further found to release Crm1 and RanGTP from Nup42 in the GTP hydrolysis assay (not shown). These data might indicate that Nup36 could replace RanBP1 function. However, the constant of RanGTP dissociation from the Crm1/RanGTP/Nup36 complex seems to be higher than from the Crm1/RanGTP/RanBP1 complex (compare Fig. 19*A* and *C*). Nup36 has been implicated in Crm1-mediated nuclear export *in vivo* (158). In addition, Nup36 and Crm1 were found in a complex, when GST-Nup36 was overexpressed in a yeast strain that contained Crm1 fused to GFP (158). Nup36 has further been shown to genetically interact with RanGEF (159). Nup36 and RanGEF were also found in a complex, when GST-Nup36 was overexpressed in *S. cerevisiae* (159). It is not clear to date whether Nup36 is a nucleoporin (46) or a nuclear factor (159) that might be transiently associated with the NPC. However, it is intriguing to speculate that Nup36 might play a role in the intranuclear phase of protein export.

Nucleotide exchange on Ran by RanGEF is thought to occur through destabilization of the Mg^{2+}-ion coordination to Ran by RanGEF, in analogy to recent findings for other small GTPases and their respective GEFs (160, 161). This results in release of the nucleotide and formation of an intermediate nucleotide free Ran/RanGEF complex (33). This Ran/RanGEF complex is then dissociated in the presence of nucleotide and Mg^{2+} (29, 33). We have shown that Crm1 and Rev stimulate dissociation of RanGEF from Ran in the presence of GTP and Mg^{2+} (Fig. 21, step 5; Fig. 20). The concentrations of GTP required for release of RanGEF from immobilized GST-Ran are much higher than GTP concentrations required for disassembly of the Ran/RanGEF complex in solution (29, 33). This might be due to the high concentrations of protein on the glutathione-beads. Crm1 and Rev might stimulate release of RanGEF by stabilizing the newly formed RanGTP. However, Crm1 and Nup42 did not stimulate release of RanGEF from Ran over the levels seen with Crm1 alone (Fig. 20, lane 5). This result was surprising, since the Nup42/Crm1/RanGTP complex is likely to have a higher affinity for RanGTP than the Rev/Crm1/RanGTP complex, as judged by the resistance of these complexes to RanGAP (Floer, M. and Blobel, G., unpublished observation). Therefore, these data might indicate that Rev and Crm1 form a complex with

RanGTP that is better protected against RanGEF than the Nup42/Crm1/RanGTP complex (Fig. 20). This would ensure that the newly formed Crm1/RanGTP complex is substrate bound, before it docks at a next nucleoporin.

A large fraction of Ran in the nucleus might be present in a complex with RanGEF (29). One could therefore speculate that dissociation of Ran from RanGEF by NES-protein bound Crm1 but not in the absence of an export substrate (Fig. 20), might be the event that initiates export. The subsequent docking and release reactions would result in the formation of complexes with increasing affinities for RanGTP (Fig. 21). The affinities we have measured in the GTP hydrolysis assay are a first approach to a quantitative treatment of these interactions. However, interactions of soluble transport factors with nucleoporins in the context of the NPC might be different than in solution. It should also be noted that local concentrations of transport factors at the NPC might be very high. The high affinity Crm1/RanGTP/RanBP1 complex that is formed when RanBP1 releases Crm1/RanGTP from the nucleoporin can be disassembled via RanGAP stimulated GTP hydrolysis. Alternatively, RanGEF can disassemble the complex by releasing GTP. Release of GTP may convert a high affinity complex into a lower affinity complex, leading to its disassembly. We propose an export

mechanism that does not require GTP hydrolysis but rather nucleotide release by RanGEF. GTP hydrolysis might occur only on the cytoplasmic side of the NPC. Accumulation of RanGTP on the cytoplasmic side of the NPC would be detrimental to protein import (72, 162), and presumably is tightly controlled.

4.4 Conclusions

We have studied interactions between several proteins involved in nuclear protein import and export *in vitro*. However, the results from our experiments must be seen in light of the subcellular localization of these proteins and their effective concentrations in the cell. One important issue that remains to be resolved concerns the concentration of RanGTP in the cell nucleus. Because of the mostly nuclear localization of RanGEF and the cytosolic localization of RanGAP it has been proposed that nuclear Ran is mostly GTP bound. However, it has yet to be examined, whether this nuclear pool of RanGTP indeed exists and whether RanGTP is freely diffusible in the nucleoplasm. Furthermore, little is known to date about RanGEF in the nucleus. RanGEF has been isolated as a chromatin binding protein. However, it remains to be determined, whether RanGEF is indeed in a complex with DNA and what the significance of such a

DNA-bound pool of RanGEF would be. A further question that is still unresolved is whether RanGEF is also localized at the NPC. To this end we have made an antibody against recombinant RanGEF, which is currently used for immunofluorescence and immuno-electronmicroscopic studies. Another important issue that remains to be investigated is the localization of different nucleoporins in *S. cerevisiae*. Although we know that Nup159 localizes to the cytoplasmic fibrils of the NPC, the localization of the other nucleoporins used in our studies remains elusive.

Our studies using GTP hydrolysis and GST-solution binding assays are a first approach to our understanding of the molecular mechanism of nuclear transport. However, it has become clear during our studies that the exact sequence of reactions is difficult to determine, since many reaction intermediates exist. To determine the exact sequence of events, the energy barriers between different reaction intermediates will have to be determined. Furthermore, the reaction intermediates we have identified are still ill defined, since the stoichiometries of many such complexes are not known. One approach to determine the stoichiometries of the different complexes we have identified would be a crystallographic analysis. Such an approach has been initiated for the karyopherin

β2 homolog and revealed interesting information on the karyopherin β2/RanGTP interaction (Chook Y.M. and Blobel G., paper in preparation). Another interesting question concerns karyopherin/nucleoporin interactions. Although many peptide repeat containing nucleoporins have been found to bind to karyopherins, it is not clear to date how a docking site on a repeat containing nucleoporin is defined. An investigation of the karyopherin/nucleoporin interactions might also provide further information on the hypothesis, that directionality in transport might at least in part be conferred by different nucleoporins that have different affinities for a specific karyopherin (see chapter 3). In summary, our studies have raised many new questions that await to be addressed.

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