

Disassembly of RanGTP-Karyopherin β Complex, an Intermediate in Nuclear Protein Import*

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We previously showed that RanGTP forms a 1:1 complex with karyopherin β that renders RanGTP inaccessible to RanGAP (Floer, M., and Blobel, G. (1996) *J. Biol. Chem.* 271, 5313–5316) and karyopherin β functionally inactive (Rexach, M., and Blobel, G. (1995) *Cell* 83, 683–692). Recycling of both factors for another round of function requires dissociation of the RanGTP-karyopherin β complex. Here we show using BIAcore™, a solution binding assay, and GTP hydrolysis and exchange assays, with yeast proteins, that karyopherin β and RanGTP are recycled efficiently in a reaction that involves karyopherin α , RanBP1, RanGAP, and the C terminus of the nucleoporin Nup1. We find that karyopherin α first releases RanGTP from karyopherin β in a reaction that does not require GTP hydrolysis. The released RanGTP is then sequestered by RanBP1, and the newly formed karyopherin $\alpha\beta$ binds to the C terminus of Nup1. Finally, RanGTP is converted to RanGDP via nucleotide hydrolysis when RanGAP is present. Conversion of RanGTP to RanGDP can also occur via nucleotide exchange in the presence of RanGEF, an excess of GDP, and if RanBP1 is absent. Additional nucleoporin domains that bind karyopherin $\alpha\beta$ stimulate recycling of karyopherin β and Ran in a manner similar to the C terminus of Nup1.

Transport of proteins that contain a nuclear localization signal (NLS)¹ into the nucleus of the cell requires energy, mobile transport factors, and nuclear pore complexes (NPC) in the nuclear envelope. Karyopherin $\alpha\beta$ heterodimer (also termed importin $\alpha\beta$, NLS receptor-p97 complex, PTAC, or Kap60/95) binds proteins that contain an NLS similar to that of the SV40 large T-antigen or nucleoplasmin (NLS protein) and brings them to the NPC (3–12). Karyopherin α binds the NLS protein (2, 3, 7, 13–15), whereas karyopherin β increases the affinity of karyopherin α for the NLS (2, 16) and docks the karyopherin α -NLS-protein complex to a subfamily of NPC proteins (nucleoporins) that contain XXFG-peptide repeats (2, 14, 17–20). The subsequent translocation across the NPC requires Ran/TC4 (21, 22) and p10/NTF2 (23, 24). p10 is a dimer

(24, 25) that binds RanGDP (26, 27) and karyopherin β (26, 28) and functions to tether RanGDP to karyopherin $\alpha\beta$ heterodimers that are docked to nucleoporins (26). When Ran is in its GTP-bound form it disrupts the interaction of karyopherin β with karyopherin α and with FXFG regions of nucleoporins by forming a complex with karyopherin β (2). The repetitive interaction of transport factors, substrates, and nucleoporins at the NPC may facilitate the transport of substrates across the NPC (2, 17).

Accessory factors regulate nuclear transport by modulating Ran. The GTPase-activating protein for Ran, RanGAP (termed RanGAP1, or Rna1 in yeast) (29–32), and the nucleotide exchange factor for Ran, RanGEF (termed RCC1, or Prp20 in yeast) (33–35), are required to sustain efficient transport of substrates across the NPC (36–39). The Ran binding protein 1, RanBP1 (40), is also involved in nuclear transport (41, 42). As the RanGTP-karyopherin β complex is resistant to stimulation of GTP hydrolysis by RanGAP (1, 39, 43), RanGAP-stimulated GTP hydrolysis cannot dissociate the RanGTP-karyopherin β complex. However, dissociation of RanGTP-karyopherin β is crucial to recycle both factors for another round of function.

We show here that the RanGTP-karyopherin β complex is disassembled in the presence of karyopherin α , the C terminus of Nup1 (C-Nup1), RanGAP, and RanBP1. A detailed analysis of the reaction mechanism revealed that karyopherin β is first released from RanGTP by karyopherin α , followed by conversion of RanGTP to RanGDP in the presence of RanGAP and RanBP1. Interaction of RanBP1 with RanGTP not only stimulates GTP hydrolysis in the presence of RanGAP but also prevents reformation of the RanGTP-karyopherin β complex in the presence of C-Nup1 and karyopherin α . C-Nup1 sequesters the released karyopherin β by forming a C-Nup1-karyopherin $\alpha\beta$ complex. Formation of this ternary complex makes rebinding of karyopherin β to RanGTP less favorable. Nup36 and a fragment containing the FXFG repeat region of Nup1 function in the disassembly reaction as well, although with lower activity than C-Nup1.

EXPERIMENTAL PROCEDURES

Protein Expression and Purification—Yeast Ran and RanGAP were expressed and purified as described (1). Yeast karyopherin α (Kap60) and karyopherin β (Kap95), the Nup1 fragment containing a FXFG repeat region (AA 432–816) and the Nup2 fragment containing a FXFG repeat region (AA 186–561), were expressed as glutathione *S*-transferase (GST) fusion proteins as described (2, 12). Proteins were purified, and the GST moiety was cleaved with thrombin as described for GST-fusion proteins (1, 2).

RanGEF, the C terminus of Nup1 (AA 963–1076) (C-Nup1), and Nup36 were expressed as GST-fusion proteins. The genes or gene fragments encoding these proteins were amplified by polymerase chain reaction from *Saccharomyces cerevisiae* genomic DNA (Promega). The RanGEF polymerase chain reaction product was inserted into vector pGEX-2TK (Pharmacia Biotech Inc.) as a *Bgl*II-*Eco*RI fragment and amplified C-Nup1 and Nup36 were inserted into pGEX-2TK as *Bam*HI-*Eco*RI fragments. The proteins were expressed in *Escherichia coli*

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¹ The abbreviations used are: NLS, nuclear localization sequence; GAP, GTPase-activating protein; GEF, guanine nucleotide exchange factor; C-Nup1, C terminus of Nup1 (amino acids 963–1076); AA, amino acids; GMP-PCP, guanylyl-(β , γ -methylene) diphosphonate; GST, glutathione *S*-transferase; RU, resonance units (44); NPC, nuclear pore complex; PAGE, polyacrylamide gel electrophoresis.

strain BLR (Novagen). The GST-fusion proteins of RanGEF and Nup36 were purified from bacterial lysates on glutathione-Sepharose beads (Pharmacia), and the GST moiety was cleaved with thrombin as described for GST-fusion proteins (1, 2). The GST-fusion protein of C-Nup1 was purified on glutathione-Sepharose beads and eluted with 10 mM glutathione, as described previously for GST-fusion proteins (12). The purified proteins were stored in frozen aliquots at -80°C .

Yeast RanBP1 (Yrb1) was amplified from *S. cerevisiae* genomic DNA (Promega) by polymerase chain reaction and inserted as a *NcoI*-*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 $2,000 \times g$, 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 dithiothreitol). 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 $10,000 \times g$ supernatant was loaded onto a MonoQ fast protein liquid chromatography column (Pharmacia), 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 Superdex 75 fast protein liquid chromatography column (Pharmacia) which was equilibrated with buffer A (150 mM KOAc, 20 mM Hepes, pH 7.3, 2 mM $\text{Mg}(\text{OAc})_2$, 1 mM dithiothreitol). RanBP1 eluted as a dimer with a mobility equal to that of a 70-kDa globular protein. Fractions containing RanBP1 were pooled and aliquots were stored at -80°C .

Solution Binding Assay.—For each experiment, an *E. coli* lysate containing GST-C-Nup1 was incubated for 20 min at 4°C with glutathione-agarose beads (Sigma) ($2 \mu\text{g}$ of C-Nup1 per $10 \mu\text{l}$ of beads) in 0.5 ml of binding buffer (20 mM Hepes, pH 6.8, 150 mM KOAc, 2 mM $\text{Mg}(\text{OAc})_2$, 1 mM dithiothreitol, 0.1% Tween 20). Beads were washed 6 times by centrifugation ($2,000 \times g$ for 30 s) and resuspension in 0.5 ml of chilled binding buffer. One-step assay: $20 \mu\text{l}$ 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 $40 \mu\text{l}$. Tubes were tumbled end over end for 1 h at 4 or 21°C as indicated in the figure legends. Two-step assay: the bead slurry was incubated for 40 min at 4°C with Kap95 ($0.6 \mu\text{g}$ for every $10 \mu\text{l}$ of beads) in 0.5 ml of binding buffer. After washing 3 times with 0.5 ml of binding buffer, beads were resuspended as a 50% slurry and incubated with protein additions as in the one-step assay. Beads were subjected to centrifugation at $2,000 \times g$ for 30 s, and unbound proteins were collected by removing $30 \mu\text{l}$ from the meniscus; this constitutes the unbound fraction. Beads were washed twice with 0.5 ml of chilled binding buffer as before, and $28 \mu\text{l}$ of binding buffer was added; this constitutes the bound fraction. 12 or $10 \mu\text{l}$ of $6 \times$ Laemmli sample buffer with 2-mercaptoethanol was added to bound and unbound fractions, respectively. After incubation at 95°C for 15 min, proteins in $18 \mu\text{l}$ of each sample were resolved by SDS-PAGE and stained with Coomassie Blue.

GTP Hydrolysis and Nucleotide Exchange Assays.—GTP hydrolysis assays were conducted as described previously (1). 15 nM Ran- $[\gamma\text{-}^{32}\text{P}]\text{GTP}$ and 25 nM karyopherin β were incubated for 10 min at 21°C , and then RanGAP, karyopherin α , C-Nup1, and RanBP1 were added as indicated in the figure legends. Reactions were incubated for 20 min at 21°C . Nucleotide exchange assays were performed essentially as GTP hydrolysis assays, except that Ran was labeled with $[\alpha\text{-}^{32}\text{P}]\text{GTP}$, and $200 \mu\text{M}$ GTP and $200 \mu\text{M}$ GDP were added to the exchange reaction. 15 nM Ran- $[\alpha\text{-}^{32}\text{P}]\text{GTP}$ was incubated with 25 nM karyopherin β for 10 min at 21°C , and then RanGEF, karyopherin α , C-Nup1, and RanBP1 were added. Reactions were incubated for 20 min at 21°C .

BIAcore™ Experiments.—The BIAcore™ methodology is described elsewhere (44). 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 $5 \mu\text{l}$ of a 1:1 mixture of 0.05 M *N*-hydroxysuccinimide and 0.2 M *N*-ethyl-*N'*-(3-dimethylaminopropyl)carbodiimide hydrochloride at a flow rate of $5 \mu\text{l}/\text{min}$. Ran was coupled by injecting $30 \mu\text{l}$ of a solution containing $10 \mu\text{g}/\text{ml}$ Ran in 10 mM NaOAc, pH 5.0, 2 mM $\text{Mg}(\text{OAc})_2$. The surface was blocked by injecting $30 \mu\text{l}$ of 300 mM Tris-HCl, pH 8.0, 0.1 mM GTP, 2 mM $\text{Mg}(\text{OAc})_2$. Typically, this resulted in immobilization of 300–400 resonance units (RU) of Ran on the sensor chip surface. We determined using a method described in Ref. 1 that only $\sim 40\%$ of the Ran was GTP-bound and $\sim 60\%$ 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 $10 \mu\text{l}/\text{min}$ at 25°C . The Ran surface was regenerated by injecting $5 \mu\text{l}$ of 0.5% Triton X-100 and 0.01% SDS in water. To obtain RanGMP-PCP, $30 \mu\text{M}$ Ran was

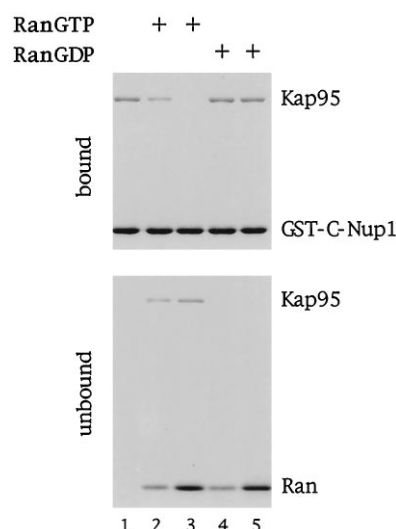


FIG. 1. Karyopherin β (Kap95) binds to the C terminus of Nup1 (C-Nup1), and binding is abolished by RanGTP but not RanGDP. Immobilized GST-C-Nup1 ($2 \mu\text{g}$ per $10 \mu\text{l}$ of packed beads) was preincubated with $0.6 \mu\text{g}$ of Kap95 for 40 min at 4°C . After washing, the beads were incubated for 40 min at 21°C with no addition (lane 1), $0.6 \mu\text{g}$ of RanGTP (lane 2), $2 \mu\text{g}$ of RanGTP (lane 3), $0.6 \mu\text{g}$ of RanGDP (lane 4), or $2 \mu\text{g}$ of RanGDP (lane 5). RanGDP and RanGTP were prepared as described (2). Bound and unbound fractions were analyzed by SDS-PAGE and Coomassie Blue staining.

incubated for 1 h at 21°C in 0.5 ml of buffer A with 5 mM EDTA, 2 mM GMP-PCP, and no $\text{Mg}(\text{OAc})_2$. 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 $\sim 39\%$ GMP-PCP, $\sim 60\%$ GDP, and less than 1% GTP, determined as described (1). A RanGMP-PCP surface was generated as described for Ran.

RESULTS

We previously showed that RanGTP forms a complex with karyopherin β (1, 2). Estimates from the inhibition of RanGAP by karyopherin β indicate an affinity below 1 nM for the RanGTP-karyopherin β interaction² (0.3 nM for mammalian proteins (39)). Here we investigated the disassembly of the yeast RanGTP-karyopherin β complex and its regulation by transport factors and nucleoporins. As RanGAP is synthetically lethal with the C terminus of the nucleoporin Nup1 (45) and with karyopherin β (46), we investigated whether the C terminus of Nup1 and RanGAP are involved in the disassembly of the RanGTP-karyopherin β complex.

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

² M. Floer and G. Blobel, unpublished data.

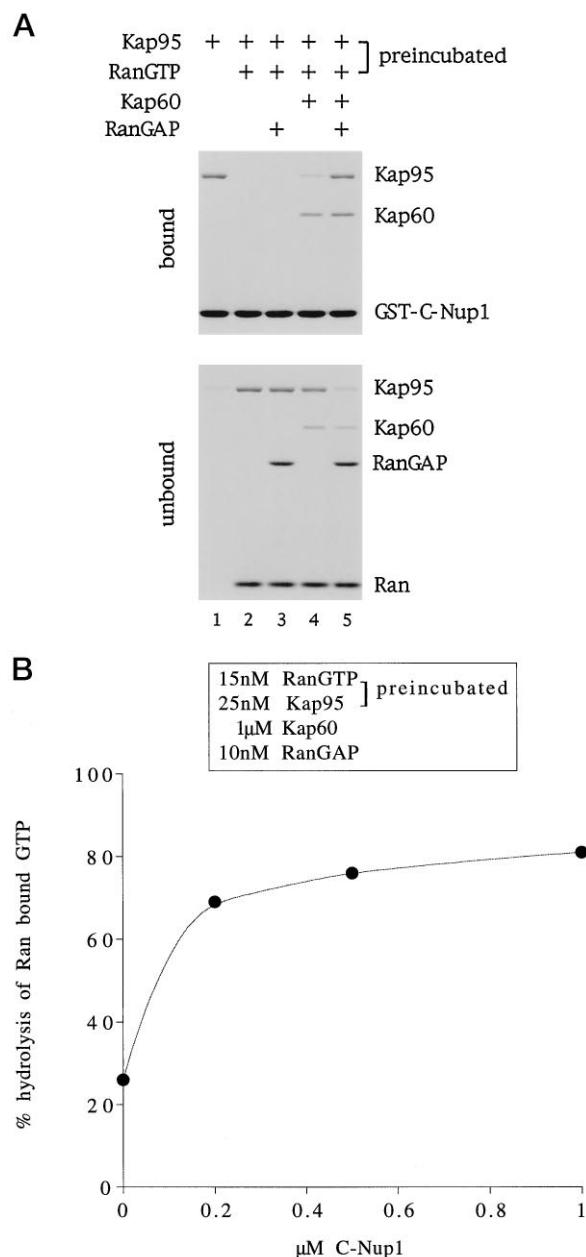


FIG. 2. The RanGTP-karyopherin β (Kap95) complex is disassembled in the presence of karyopherin α (Kap60), RanGAP, and the C terminus of Nup1 (C-Nup1). A, immobilized GST-C-Nup1 (2 μ g per 10 μ l of packed beads) was incubated with 0.6 μ g of Kap95 (lane 1) or 0.6 μ g of Kap95 that had been preincubated with 1 μ g of RanGTP for 15 min at 4 $^{\circ}$ C (lanes 2–5). RanGTP was prepared as described (2). Reactions also contained 1 μ g of RanGAP (lanes 3 and 5) and 0.6 μ g of Kap60 (lanes 4 and 5). Reactions were incubated for 45 min at 21 $^{\circ}$ C and then for 15 min at 4 $^{\circ}$ C. Bound and unbound fractions were analyzed by SDS-PAGE and Coomassie Blue staining. B, GTP hydrolysis assays were performed as described under “Experimental Procedures.” 15 nM Ran-[γ - 32 P]GTP was preincubated with 25 nM Kap95 for 10 min at 21 $^{\circ}$ C. Then 10 nM RanGAP, 1 μ M Kap60, and increasing amounts of C-Nup1 were added. Reactions were incubated for 20 min at 21 $^{\circ}$ C. The extent of GTP hydrolysis was quantified as described under “Experimental Procedures.”

will be discussed elsewhere.³ Most importantly, when karyopherin α and RanGAP were added together, karyopherin β binding to C-Nup1 was restored (lane 5). These results indicate that the RanGTP-karyopherin β complex is disrupted in the presence of karyopherin α , C-Nup1, and RanGAP and that

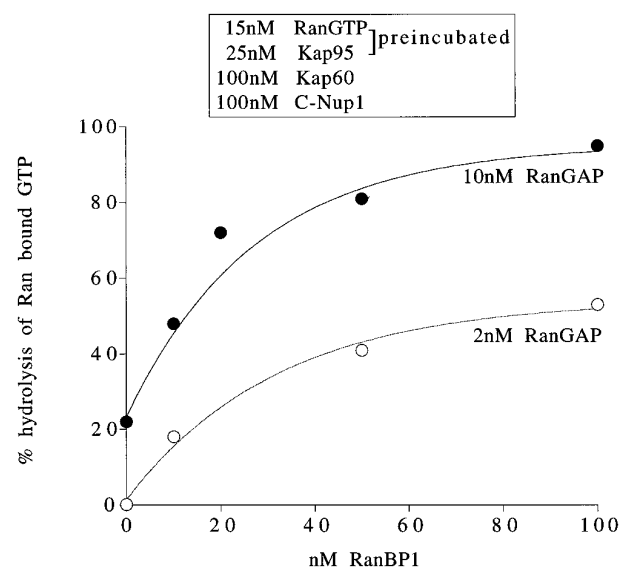


FIG. 3. RanBP1 stimulates GTP hydrolysis in the presence of karyopherin β (Kap95), RanGAP, karyopherin α (Kap60), and the C terminus of Nup1 (C-Nup1). GTP hydrolysis assays were performed as described under “Experimental Procedures.” 15 nM Ran-[γ - 32 P]GTP was preincubated with 25 nM Kap95 for 10 min at 21 $^{\circ}$ C. Then 100 nM Kap60, 100 nM C-Nup1, 10 nM RanGAP (closed circles) or 2 nM RanGAP (open circles), and increasing amounts of RanBP1 were added. Reactions were incubated for 20 min at 21 $^{\circ}$ C. The extent of GTP hydrolysis was quantified as described under “Experimental Procedures.”

RanGTP is converted to RanGDP through GTP hydrolysis stimulated by RanGAP.

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 β (not shown) (1). However, addition of 1 μ M karyopherin α and 10 nM RanGAP to a mixture of 15 nM RanGTP and 25 nM karyopherin β that had been preincubated resulted in 25% GTP hydrolysis (Fig. 2B). If 200 nM C-Nup1 was added to this reaction, 70% of the Ran-bound GTP was hydrolyzed, and if 1 μ M 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 β and that this reaction is stimulated by C-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 (40, 47) and enhances RanGAP-stimulated GTP hydrolysis by Ran (41, 43, 47) (Fig. 9). Addition of RanBP1 and RanGAP to a mixture of RanGTP and karyopherin β that had been preincubated did not promote GTP hydrolysis (not shown). However, when RanBP1 and RanGAP were added together with karyopherin α and C-Nup1, GTP hydrolysis was greatly stimulated (Fig. 3). In a control reaction that contained 15 nM RanGTP and 25 nM karyopherin β 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. 3, 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. Noticeably, the concentration of RanGAP required was lower in the presence of RanBP1 (open symbols); this is consistent with previous findings on RanBP1 function in the absence of karyopherin β (41, 47) (Fig. 9). These results demonstrate that RanGTP-karyopherin β complex is fully disassembled in a reaction that requires karyopherin α and is stimulated by RanGAP, RanBP1, and the C terminus of Nup1.

³ M. Rexach, and G. Blobel, submitted for publication.

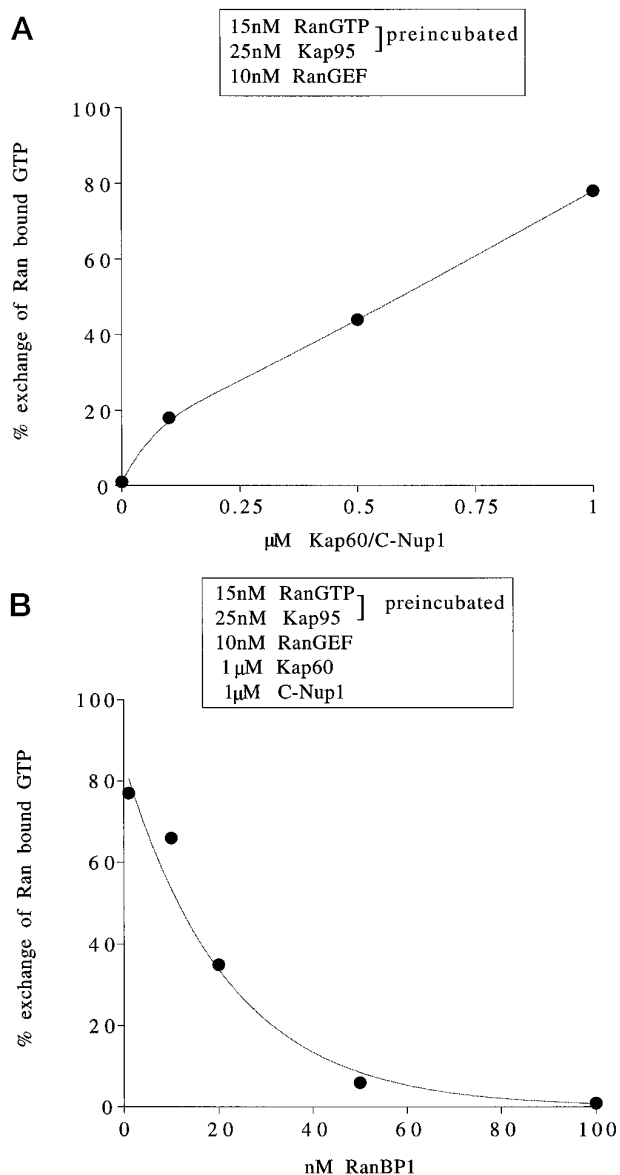


FIG. 4. RanGEF can replace RanGAP in disassembly of RanGTP-karyopherin β (Kap95) complex; RanGEF is inhibited by RanBP1. Guanine nucleotide exchange assays were performed as described under "Experimental Procedures." 15 nM Ran- $[\alpha\text{-}^{32}\text{P}]\text{GTP}$ was preincubated with 25 nM karyopherin β for 10 min at 21 °C. **A**, 10 nM RanGEF, 200 μM GDP, 200 μM GTP, and increasing amounts of Kap60 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 Kap60, 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 under "Experimental Procedures."

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 (48). Due to the higher concentration of GTP in the cell (49), it is generally assumed that RanGEF exchanges Ran-bound GDP for GTP *in vivo* (48). Nevertheless, RanGEF can exchange Ran-bound GTP for GDP *in vitro* if an excess of GDP is provided (48). Although recombinant RanGEF stimulated nucleotide exchange on Ran (not shown), it was incapable of stimulating exchange when RanGTP was bound to karyopherin β (Fig. 4A) (39). 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 β that had been preincubated (Fig. 4A). 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 (not shown). We also tested the effect of RanBP1 on this 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 β that had been preincubated (Fig. 4B). This result is consistent with previous reports on the inhibitory effect of RanBP1 on the exchange of Ran-bound GTP when karyopherin β is absent (43, 47).

To analyze further the effect of RanGEF and RanBP1 on the disassembly of RanGTP-karyopherin β complex, we carried out solution binding assays similar to those described in Fig. 2A. As expected, RanGEF could replace RanGAP (Fig. 5A) 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 β binding to C-Nup1 (lane 6). This result suggests that RanGTP is accessible to RanGAP, or RanGEF, after the RanGTP-karyopherin β 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); we are currently investigating the significance of this interaction. We then tested whether inhibition of RanGEF function by RanBP1 had an effect on RanGTP-karyopherin β disassembly. Unexpectedly, disassembly occurred even in the presence of RanBP1 (compare lane 7 to lane 4). This surprising result suggests that RanBP1 can directly stimulate disassembly of the RanGTP-karyopherin β complex in the presence of karyopherin α and C-Nup1. Indeed RanBP1 stimulated disassembly in the presence of karyopherin α and C-Nup1 (Fig. 5B, lane 4) but not in the absence of karyopherin α (lane 5). Thus karyopherin α is required to disrupt the RanGTP-karyopherin β complex in the presence of RanBP1 and C-Nup1. Neither RanBP1 nor RanGTP bound to the karyopherin $\alpha\beta$ -C-Nup1 complex (not shown). These results suggest that RanBP1 stimulates disassembly of the RanGTP-karyopherin β 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 had been released from karyopherin β , since RanBP1 binds RanGTP (not shown) (40, 43, 47).

To analyze in detail the mechanism of RanGTP-karyopherin β disassembly we used BIAcore™ (44) 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 under "Experimental Procedures." 100 $\mu\text{g}/\text{ml}$ karyopherin β was injected for 3 min at a flow rate of 10 $\mu\text{l}/\text{min}$ over the Ran surface which typically resulted in binding of 500 resonance units (RU) of karyopherin β (Fig. 6A). This was followed by a wash-out phase of 3 min to monitor karyopherin β dissociation. We then examined whether the dissociation rate of the RanGTP-karyopherin β complex increases when other proteins are injected during the wash-out phase. For these experiments 100–200 RU of karyopherin β 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 β from RanGTP (Fig. 6B). The amount released was dependent on the concentration of karyopherin α injected with a linear relation between released karyopherin β and the concentration of karyopherin α

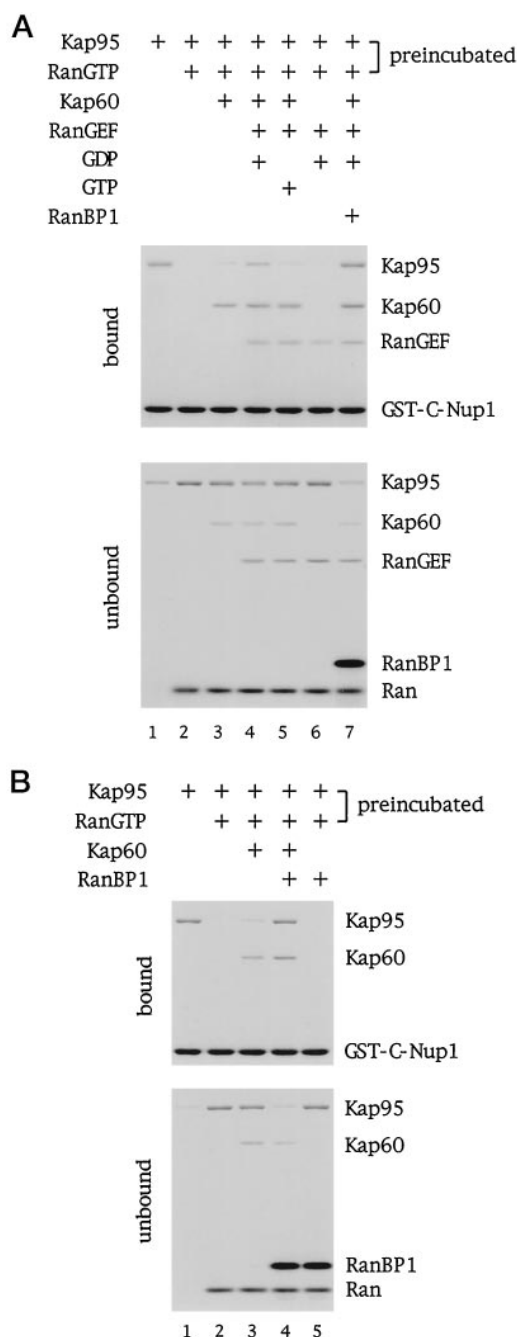


FIG. 5. RanBP1 stimulates RanGTP-karyopherin β (Kap95) disassembly in the presence of karyopherin α (Kap60), the C terminus of Nup1 (C-Nup1), and in the presence and absence of RanGEF. A, immobilized GST-C-Nup1 (2 μ g per 10 μ l of packed beads) was incubated with 0.6 μ g of Kap95 (lane 1) or 0.6 μ g of Kap95 that had been preincubated with 1 μ g of RanGTP for 15 min at 4 $^{\circ}$ C (lanes 2–7). RanGTP was prepared as described (2). Reactions also contained 0.6 μ g of Kap60 (lanes 3–5 and 7), 0.4 μ g of RanGEF (lanes 4–7), 250 μ M GDP (lanes 4, 6 and 7), 250 μ M GTP (lane 5), and 2 μ g of RanBP1 (lane 7). Reactions were incubated for 45 min at 21 $^{\circ}$ C and then for 15 min at 4 $^{\circ}$ C. Bound and unbound fractions were analyzed by SDS-PAGE and Coomassie Blue staining. B, immobilized GST-C-Nup1 (2 μ g per 10 μ l of packed beads) was incubated with 0.6 μ g of Kap95 (lane 1) or 0.6 μ g of Kap95 that had been preincubated with 1 μ g of RanGTP for 15 min at 4 $^{\circ}$ C (lanes 2–5). Reactions also contained 0.6 μ g of Kap60 (lanes 3 and 4) and 2 μ g of RanBP1 (lanes 4 and 5). Reactions were incubated for 1 h at 4 $^{\circ}$ C. Bound and unbound fractions were analyzed by SDS-PAGE and Coomassie Blue staining.

(0–2 μ g/ml karyopherin α) (Fig. 6B, inset). This result indicates that karyopherin α disassembles the RanGTP-karyopherin β complex and that this disassembly is a first order reaction with

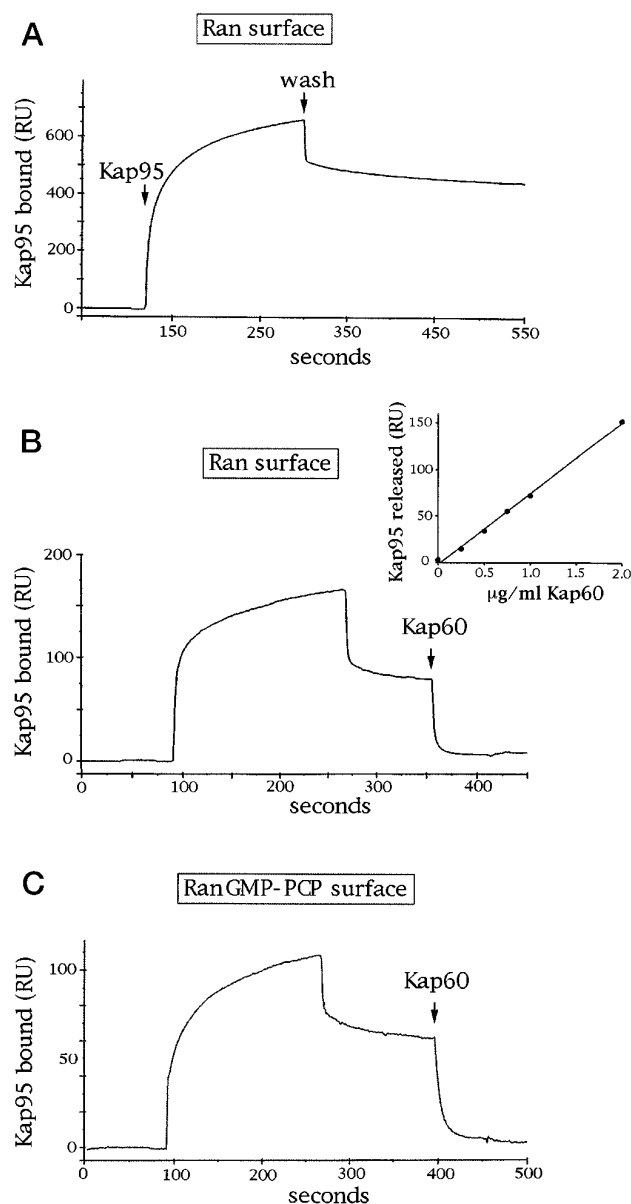


FIG. 6. Karyopherin α (Kap60) releases karyopherin β (Kap95) from RanGTP. A, Ran that was \sim 40% GTP and \sim 60% GDP bound was immobilized on a CM5 sensor chip as described under “Experimental Procedures.” Injection of 100 μ g/ml Kap95 resulted in binding of 500 RU to the Ran surface. B, Ran was immobilized as in A, and 90 RU of Kap95 were bound to the Ran surface. 1 μ g/ml Kap60 was injected for 1 min during the wash-out phase. The inset shows release of Kap95 by various concentrations of Kap60. The amount of Kap95 released was plotted against the concentration of Kap60 used. C, Ran that was \sim 39% GMP-PCP, \sim 60% GDP, and $<$ 1% GTP bound was immobilized as described under “Experimental Procedures.” 70 RU of Kap95 were bound to the surface, and 2 μ g/ml Kap60 was injected for 1 min during the wash-out phase.

respect to karyopherin α . The apparent rate of RanGTP-karyopherin β dissociation in the presence of saturating concentrations of karyopherin α was faster than the detection limit of BIAcoreTM (>0.1 s⁻¹); hence, stimulation of RanGTP-karyopherin β dissociation by karyopherin α could not be measured directly. The rate of RanGTP-karyopherin β dissociation in the absence of karyopherin α was calculated to be 4.5×10^{-4} s⁻¹ based on the conditions of these experiments. We therefore estimate that karyopherin α stimulates dissociation of the RanGTP-karyopherin β complex by at least 3 orders of magnitude. RanGAP did not increase the amount of karyopherin β

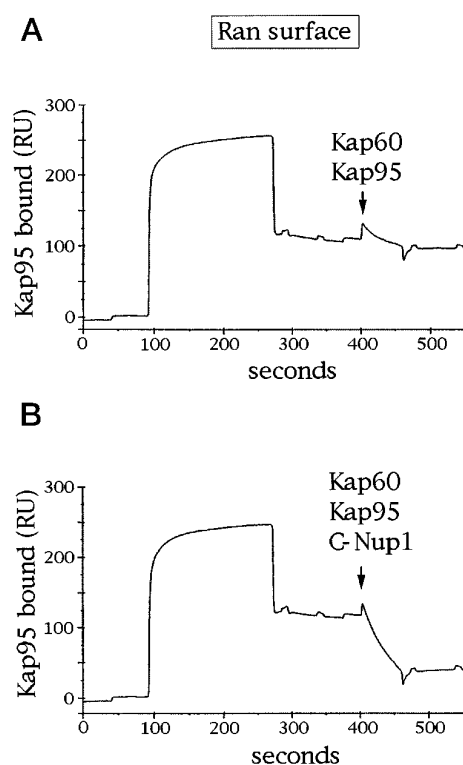


FIG. 7. The C terminus of Nup1 (C-Nup1) sequesters karyopherin $\alpha\beta$ (Kap60/95) and prevents reformation of the RanGTP-karyopherin β complex. A, 120 RU of karyopherin β was bound to a Ran surface prepared with Ran containing $\sim 40\%$ GTP and $\sim 60\%$ GDP as described under "Experimental Procedures." During the wash-out phase a solution containing 35 nM Kap60 and 5 nM Kap95 was injected for 1 min. B, 120 RU of Kap95 was bound to the Ran surface as in A. During the wash-out phase a solution containing 35 nM C-Nup1, 5 nM Kap95, and 35 nM Kap60 was injected for 1 min.

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 β .

To test whether intrinsic GTP hydrolysis by Ran is a prerequisite for karyopherin α -induced dissociation of karyopherin β , we used RanGMP-PCP instead of RanGTP; GMP-PCP is a non-hydrolyzable analog of GTP. Ran was incubated with GMP-PCP as described under "Experimental Procedures" to obtain Ran that was $\sim 39\%$ GMP-PCP, $\sim 60\%$ GDP, and less than 1% GTP-bound. RanGMP-PCP was immobilized on a CM5 sensor chip as described for Ran. Karyopherin β bound to RanGMP-PCP with the same apparent kinetics as to RanGTP under these conditions (compare Fig. 6, C to A). When karyopherin α was injected during the wash-out phase, all the karyopherin β was released (Fig. 6C). The release of karyopherin β from RanGMP-PCP showed the same dependence on the concentration of karyopherin α as release of karyopherin β from RanGTP (not shown). This result demonstrates that GTP hydrolysis is not required for the karyopherin α -dependent dissociation of RanGTP from karyopherin β .

We also used BIAcoreTM to investigate the role of C-Nup1 in the disassembly of the RanGTP-karyopherin β complex. C-Nup1 did not release karyopherin β 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 β complex by karyopherin α , as judged by the GTP hydrolysis assay (Fig. 2B), yet does not stimulate RanGAP activity directly (not shown). To understand

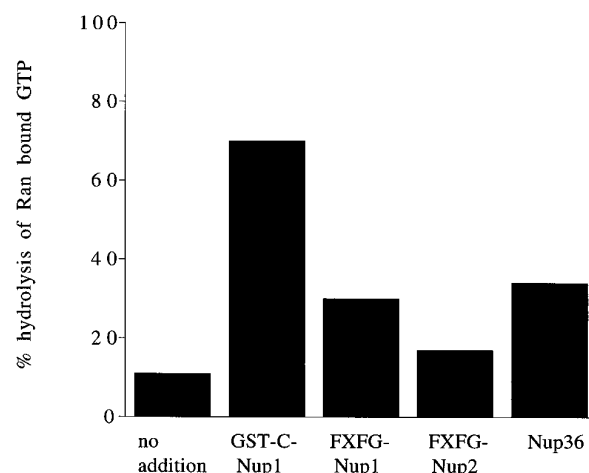


FIG. 8. RanGTP-karyopherin β (Kap95) disassembly is promoted by different fragments of nucleoporins that bind karyopherin $\alpha\beta$ (Kap60/95). GTP hydrolysis assays were performed as described under "Experimental Procedures." 15 nM Ran- $[\gamma\text{-}^{32}\text{P}]\text{GTP}$ was preincubated with 25 nM karyopherin β for 10 min at 21 $^{\circ}\text{C}$. 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 at 21 $^{\circ}\text{C}$ for 20 min. The extent of GTP hydrolysis was quantified as described under "Experimental Procedures."

the role of C-Nup1 in the RanGTP-karyopherin β disassembly reaction, we compared the GTP hydrolysis and BIAcoreTM experiments. In the BIAcoreTM 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 β may rebind to RanGTP before RanGAP stimulates GTP hydrolysis, whereas in the BIAcoreTM experiment the released karyopherin β is removed by constant flow and cannot rebind to RanGTP. In BIAcoreTM rebinding during the wash-out phase is significant only when high density surfaces and low flow rates are used (50). As there was only ~ 160 RU of RanGTP immobilized on the surface, and there was no change in the RanGTP-karyopherin β dissociation rate at flow rates of up to 30 $\mu\text{l}/\text{min}$, we assume that rebinding of karyopherin β to RanGTP did not occur. To test whether C-Nup1 affects rebinding of karyopherin β to RanGTP, we coinjected karyopherin β during the wash-out phase; this retards the diffusion of dissociated karyopherin β from the Ran surface and may promote rebinding of karyopherin β before it is removed by the wash. When 5 nM karyopherin β was coinjected with 35 nM karyopherin α (2 $\mu\text{g}/\text{ml}$), release of karyopherin β was completely inhibited (Fig. 7A). Strikingly, release of karyopherin β from the RanGTP surface was restored when 35 nM C-Nup1 was coinjected with 35 nM karyopherin α and 5 nM karyopherin β (Fig. 7B). This result indicates that C-Nup1 sequesters karyopherin $\alpha\beta$ and prevents reformation of the RanGTP-karyopherin β complex.

As yeast karyopherin $\alpha\beta$ also binds to the FXFG repeat region of Nup1 (2), Nup2 (2), and Nup36 (26), we tested whether these nucleoporins or fragments thereof could replace C-Nup1 in the disassembly of the RanGTP-karyopherin β complex using the GTP hydrolysis assay. As a control, addition of 0.75 μM karyopherin α and 10 nM RanGAP to 15 nM RanGTP and 25 nM karyopherin β that had been preincubated resulted in 11% hydrolysis of the Ran-bound GTP (Fig. 8). Addition of 1 μM C-Nup1 to this mixture resulted in 70% hydrolysis. When 1 μM FXFG-Nup1 was added instead of C-Nup1, 34% of the Ran-bound GTP was hydrolyzed. Likewise, addition of 1 μM FXFG-Nup2 resulted in 15% hydrolysis, and addition of 1 μM full-length Nup36 resulted in 38% hydrolysis. These findings demonstrate that the FXFG repeat region of Nup1 and full-

length Nup36 can efficiently stimulate the RanGTP-karyopherin β disassembly reaction.

Nup36, in addition to binding karyopherin $\alpha\beta$ (26), also binds RanGTP⁴ (51) presumably through a Ran-binding domain in its C terminus (26, 51–53). We find that Nup36 can stimulate GAP activity in a manner similar to RanBP1 (Fig. 9) (51). Addition of 1 μ M Nup36 (*open squares*) resulted in greater stimulation of GAP activity than addition of 1 μ M RanBP1 (*closed circles*). This result suggests that Nup36 functions in the RanGTP-karyopherin β disassembly reaction by stimulating conversion of RanGTP to RanGDP via GTP hydrolysis in the presence of RanGAP, as well as by sequestering karyopherin $\alpha\beta$.

DISCUSSION

Our results suggest a model for RanGTP-karyopherin β disassembly (Fig. 10). First, karyopherin β is released from RanGTP by karyopherin α in a reaction that does not require GTP hydrolysis (Fig. 10, *step 1*; Fig. 6). RanGTP is then bound by RanBP1 which prevents reformation of the RanGTP-karyopherin β complex in the presence of C-Nup1 and karyopherin α (Fig. 10, *step 2*; Fig. 5B). RanGTP bound to RanBP1 has a higher affinity for RanGAP than unbound RanGTP, so that conversion of RanGTP to RanGDP is enhanced (Fig. 10, *step 3*; Figs. 3 and 9) (41, 43, 47). RanBP1 binding to RanGTP also prevents interaction of RanGTP with RanGEF (Fig. 10, *step 4*; Fig. 4B) (43, 47). The newly formed karyopherin $\alpha\beta$ binds to C-Nup1 and makes rebinding of karyopherin β to RanGTP less favorable (Fig. 10, *step 5*; Figs. 2A, 5, and 7). Nup36 and the FXFG repeat region of Nup1 can also function in this step of the reaction (Fig. 8). Additional factors may modulate the disassembly reaction (*e.g.* p10 (26) or Dis3 (54)).

Our data on RanGTP-karyopherin β disassembly *in vitro* explains previously reported genetic interactions in *S. cerevisiae* between RanGAP, karyopherin β , and the C terminus of Nup1 (45, 46). A mutant form of RanGAP is synthetically lethal with a mutant form of Nup1 that lacks the C terminus (45). Our data can explain this synergistic effect as RanGAP and C-Nup1 cooperate to dissociate the RanGTP-karyopherin β complex (Fig. 2) 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 β (46). This genetic interaction can be explained as well, since our data show that RanGAP promotes recycling of karyopherin β by stimulating RanGTP-karyopherin β disassembly (Fig. 2).

RanGTP-karyopherin β complex is disassembled by karyopherin α through active release as karyopherin α increases the rate of RanGTP-karyopherin β dissociation (Fig. 6B). Release of karyopherin β from RanGTP is linearly dependent on the concentration of karyopherin α (Fig. 6B, *inset*); this indicates that RanGTP-karyopherin β disassembly is a first order reaction with respect to karyopherin α . We estimate that karyopherin α stimulates RanGTP-karyopherin β dissociation by at least 3 orders of magnitude. Release of RanGTP from karyopherin β presumably occurs through formation of an intermediate RanGTP-karyopherin β -karyopherin 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 BIAcore™. The proposed displacement mechanism is supported by data that demonstrate that RanGTP and karyopherin α have partially overlapping binding sites on karyopherin β (55–57). Thus karyopherin α could interact with the RanGTP-karyopherin β complex through that part of its binding site on karyopherin β that is not occupied by RanGTP; this interaction might then displace RanGTP from the overlapping site. The displacement reaction is reversible as

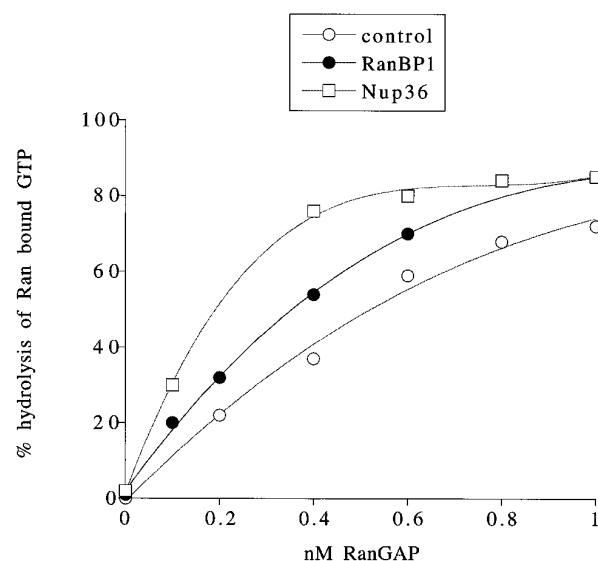


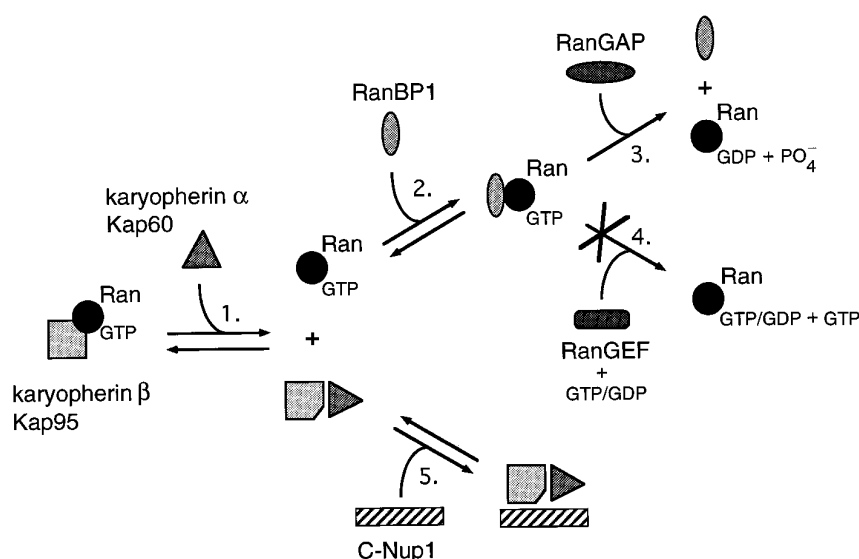
Fig. 9. **Nup36 stimulates GTP hydrolysis in the presence of RanGAP.** GTP hydrolysis assays were conducted as described under "Experimental Procedures." 15 nM Ran-[γ -³²P]GTP was incubated for 10 min at 21 °C in the presence of 1 μ M RanBP1 (*closed circles*), 1 μ M Nup36 (*open squares*), or no addition (*open circles*). Then increasing amounts of RanGAP were added followed by incubation for 20 min at 21 °C.

RanGTP dissociates karyopherin α from karyopherin β (2, 28), presumably by forming the same intermediate ternary complex. However, karyopherin β preferentially binds to RanGTP over karyopherin α (2, 55). This difference in affinity would force the RanGTP-karyopherin β disassembly reaction in the direction of RanGTP-karyopherin β complex formation (Fig. 10, *step 1*). However, in the presence of C-Nup1, RanGAP, and RanBP1, the equilibrium is shifted toward formation of the karyopherin $\alpha\beta$ complex (Fig. 2, 3, and 5B). The presence of a GST-NLS fusion protein did not affect the disassembly reaction when tested in the GTP hydrolysis or BIAcore™ experiments.²

GTP hydrolysis is not required for the release of karyopherin β from RanGTP by karyopherin α ; this is evidenced by the fact that release of karyopherin β from RanGMP-PCP occurs with the same efficiency as release from RanGTP (Fig. 6C). 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 (58). 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 (59)) 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 (60), the intrinsic GTP hydrolysis is thought to trigger complex disassembly (59). Our results on RanGTP-karyopherin β 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 β occurs much faster than the intrinsic hydrolysis of Ran-bound GTP ($k_{\text{release}} > 0.1 \text{ s}^{-1}$ compared with $k_{\text{cat}} = 5 \times 10^{-5} \text{ s}^{-1}$ (32)). Our results also suggest that RanGTP-karyopherin β dissociation in the absence of karyopherin α does not require GTP hydrolysis, as dissociation of the RanGMP-PCP-karyopherin β complex occurred with the same apparent kinetics as the dissociation of the RanGTP-karyopherin β complex under the conditions described² (Fig. 6). We are currently investigat-

⁴ M. Floer, U. Nehrbass, and G. Blobel, unpublished data.

FIG. 10. Mechanism of disassembly of the RanGTP-karyopherin β (Kap95) complex. Karyopherin α (Kap60) releases RanGTP from karyopherin β (step 1). RanBP1 then binds RanGTP and prevents reformation of the RanGTP-karyopherin β 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). Interaction of RanGTP with RanGEF is inhibited by RanBP1 (step 4). The newly formed karyopherin $\alpha\beta$ binds to the C-Nup1 and inhibits rebinding of karyopherin β to RanGTP (step 5).



ing the exact rates of association and dissociation. We propose that GTP hydrolysis during NLS-protein transport across the NPC is required to recycle Ran and karyopherin β so that each can perform multiple rounds of function.

RanGAP stimulates the disassembly of the RanGTP-karyopherin β complex (Fig. 2) by converting RanGTP to RanGDP after the karyopherin α -dependent release of RanGTP from karyopherin β thereby preventing reformation of the complex. We found that RanGEF can replace RanGAP in the disassembly reaction when GDP is present (Fig. 4A). RanGAP, apart from being a cytosolic protein (61), is also localized at the NPC (38, 62, 63) and in the nucleoplasm (64). In contrast, RanGEF is located mainly in the nucleoplasm bound to chromatin (65, 66), although it may also bind to nucleoporins² (Fig. 5A). Disassembly of RanGTP-karyopherin β *in vivo* may occur in the presence of both RanGAP and RanGEF at the NPC. However, due to the higher concentration of GTP *versus* GDP in the cell (49), conversion of RanGTP to RanGDP via RanGEF is probably not efficient. It is therefore likely that *in vivo* only RanGAP is responsible for conversion of RanGTP to RanGDP.

Our results suggest that RanBP1 has three functions. First, RanBP1 binds to RanGTP after its release from karyopherin β by karyopherin α and prevents reformation of the RanGTP-karyopherin β complex when C-Nup1 is present (Fig. 5B). Second, RanBP1 interaction with RanGTP increases the affinity of RanGTP for RanGAP which results in stimulation of GAP activity (Figs. 3 and 9) (41, 43, 47). Third, RanBP1 binding to RanGTP prevents interaction of RanGTP with RanGEF (Fig. 4B) (43, 47) which might be advantageous *in vivo* to prevent futile exchange of Ran-bound GTP for GDP. RanBP1, RanGTP, and karyopherin β form a trimeric complex (42, 43, 67, 68) which may assemble during release of RanGTP from karyopherin β . However, in the presence of karyopherin α and C-Nup1 this complex is unstable as the majority of karyopherin β bound to C-Nup1 without associated RanGTP and RanBP1 (Fig. 5B). We propose that RanBP1 functions to promote recycling of RanGTP and karyopherin β at the NPC. Our proposal is consistent with the localization of RanBP1 at the nuclear envelope (41) and its proposed function in protein import (41, 42).

The C terminus of Nup1 binds karyopherin $\alpha\beta$ and stimulates RanGTP-karyopherin β disassembly (Fig. 2B). We suggest that C-Nup1 sequesters karyopherin β and inhibits reformation of the RanGTP-karyopherin β complex (Fig. 7). We also suggest that Nup36 and the FXFG repeat region of Nup1 may function in a manner similar to C-Nup1 (Fig. 8) because they also bind karyopherin $\alpha\beta$ (2, 26). The FXFG repeat region of

Nup2 binds karyopherin $\alpha\beta$ weakly (not shown) and did not significantly stimulate RanGTP-karyopherin β disassembly (Fig. 8).

Nup36 was initially identified as a karyopherin $\alpha\beta$ binding protein (26) and was later shown to bind RanGTP⁴ (51). Overexpression of a tagged version of Nup36 results in its localization to the nucleoplasm (51). However, Nup36 localizes to the nuclear envelope as visualized by immunofluorescence using antibodies against Nup36.⁵ In addition to a karyopherin $\alpha\beta$ binding site, Nup36 has a Ran-binding domain similar to the one in RanBP1 (52, 53). Nup36 stimulates GTP hydrolysis by RanGAP as does RanBP1 (Fig. 9) (51) and is synthetically lethal with RanGAP (51). This genetic interaction can be explained by our *in vitro* data as both Nup36 and RanGAP cooperate to recycle Ran and karyopherin β for another round of function. Other nucleoporins, like the mammalian Nup358 (69, 70) and yeast Nup2 (52, 53), 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 β complex has interesting implications for protein transport into the nucleus. Nucleoporins that bind karyopherin $\alpha\beta$ have different activities in the disassembly of the RanGTP-karyopherin β complex via karyopherin α (Fig. 8). This may be due to different affinities of the nucleoporins for karyopherin $\alpha\beta$. If nucleoporins are localized along the NPC with increasing affinities for karyopherin $\alpha\beta$ (from cytoplasmic to nucleoplasmic sites), disassembly of RanGTP-karyopherin β complexes and concomitant docking of karyopherin $\alpha\beta$ might occur along an affinity gradient. This affinity gradient may confer directionality to movement of transport factors and substrates across the nuclear pore complex.

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