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The Nuclear Transport Factor Karyopherin β Binds Stoichiometrically to Ran-GTP and Inhibits the Ran GTPase Activating Protein*

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The heterodimeric karyopherin functions in targeting a nuclear localization sequence (NLS)-containing protein to the nuclear pore complex followed by Ran-GTP and p10-mediated translocation of the NLS protein into the nucleoplasm. It was shown recently that Ran-GTP dissociated the karyopherin heterodimer and, in doing so, associated with karyopherin β (Rexach, M., and Blobel, G. (1995) *Cell* 83, 683–692). We show here, using all recombinant yeast proteins expressed in *Escherichia coli*, that karyopherin β binds to Ran-GTP and inhibits GTP hydrolysis stimulated by RanGAP (the Ran-specific GTPase activating protein). Inhibition of RanGAP-stimulated GTP hydrolysis by karyopherin β was dependent on karyopherin β concentration relative to Ran-GTP. Complete inhibition of RanGAP was observed at karyopherin β concentrations that were equimolar to Ran-GTP. In gel filtration experiments, we found Ran-GTP and karyopherin β to form a stoichiometric complex. Ran-GDP bound only weakly to karyopherin β. We propose that stoichiometric complex formation between karyopherin β and Ran-GTP renders Ran-GTP inaccessible to RanGAP.

Several soluble transport factors are required for import of a nuclear localization sequence (NLS)[1]-containing protein into nuclei of digitonin-permeabilized mammalian cells. Binding of the NLS protein to the nuclear rim of digitonin-permeabilized cells is mediated by a heterodimeric complex, termed karyopherin (1, 2), or nuclear pore-targeting complex (3) (karyopherin α is synonymous with NLS receptor (4), importin (5, 6), importin 60 (7), or importin α (8); karyopherin β (1) is synonymous with p97 (4, 9), importin 90 (7), or importin β (8)). Transport of the NLS protein from the nuclear rim into the nucleus requires the Ras-related GTPase Ran (10, 11) and a Ran-interactive homodimeric complex of a protein of 15 kDa that migrates in SDS-PAGE as a protein of 10 kDa and therefore has been termed p10 (12) (p10 is synonymous with NTF 2 (13)). These four transport factors are able to substitute for the requirement of cytosol in import of an NLS protein into nuclei of digitonin-permeabilized cells, even when prepared from *Escherichia coli* as recombinant proteins (14). Nevertheless, it is likely that additional soluble factors are required. These factors may remain with the digitonin-permeabilized cells in sufficient quantities and therefore not be limiting or they may perform subtle regulatory tasks that have not yet been detected with this assay. Among these factors might be several Ran regulatory proteins, such as a guanine nucleotide exchange factor (15, 16), or a GTPase activating protein (GAP) (17), or soluble Ran-binding proteins (18).

A heterodimeric karyopherin has also been isolated from the yeast *Saccharomyces cerevisiae*, and recombinant karyopherin α and β subunits have been shown to be able to dock NLS protein at the nuclear rim of digitonin-permeabilized mammalian cells (19). Moreover, it was shown that Ran-GTP (but not Ran-GDP) dissociated the karyopherin heterodimer and associated with karyopherin β although the stoichiometry of this association was not determined (20). Ran-GTP-mediated dissociation of the karyopherin heterodimer did not require GTP hydrolysis (20). An association of Ran-GTP with karyopherin β might affect the responsiveness of Ran-GTP to Ran regulatory proteins. As a RanGAP has recently been identified (17) and in yeast is the product of the *RNA1* gene (21, 22), we have tested whether association of karyopherin β with Ran-GTP modifies the response of Ran-GTP to RanGAP. Using all recombinant proteins, we found that karyopherin β inhibited RanGAP-stimulated Ran-GTP hydrolysis in a concentration-dependent manner, yielding complete inhibition at karyopherin β concentrations that are stoichiometric to Ran-GTP. In gel filtration experiments, we found that karyopherin β was capable of forming a stoichiometric complex with Ran-GTP but of binding only weakly to Ran-GDP.

EXPERIMENTAL PROCEDURES

Cloning and Purification of Recombinant Yeast Ran (Gsp1)—The *GSP1* gene (23) was amplified from *S. cerevisiae* genomic DNA (Promega) using 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 polymerase chain reaction 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 ampicillin at 37 °C to a cell density of 0.6 A_{600} unit. Isopropyl-1-thio-β-D-galactopyranoside was added to 0.1 mM to induce expression of the protein. After 3 h, cells were harvested by centrifugation at 2,000 × *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 phenylmethylsulfonyl fluoride to 0.1 mM. All subsequent operations were carried out at 4 °C. Cells were lysed in a French pressure cell at 9,000 psi, and the lysate was centrifuged for 20 min at 10,000 × *g*. Ran was precipitated from the supernatant at 25–55% ammonium sulfate saturation and centrifuged at 10,000 × *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 × *g*, the cleared supernatant was subjected to chromatography on a Mono Q FPLC column (Pharmacia Biotech Inc.) at a flow rate of 1 ml/min using a linear gradient from 0–500 mM NaCl in Tris buffer. Ran eluted between 230 and 280 mM NaCl as assayed by SDS-PAGE.

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[1] The abbreviations used are: NLS, nuclear localization sequence; GAP, GTPase activating protein; PAGE, polyacrylamide gel electrophoresis; DTT, dithiothreitol; FPLC, fast protein liquid chromatography; GST, glutathione *S*-transferase; PBS, phosphate-buffered saline.

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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 Superdex 75 FPLC column (HR 30/10; Pharmacia Biotech Inc.) equilibrated with buffer A (20 mM Hepes, pH 7.3, 110 mM KOAc, 2 mM MgOAc, 1 mM EGTA, and 2 mM DTT). Peak fractions containing 1–2 mg/ml Ran were pooled (~2 ml), and 20-μl aliquots were frozen in liquid nitrogen and stored at −80 °C.

Determination of GTP/GDP Bound to Recombinant Ran (Gsp1)—Aliquots of frozen Ran solution were applied to a Beckman System Gold and a C18 reversed-phase column (4.6 mm × 15 cm; Rainin) equilibrated with buffer B (100 mM triethylamine, pH 6.5, phosphoric acid). Using a gradient from 0–20% acetonitrile in buffer B 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}.

Purification of Yeast RanGAP (Rna1)—The *RNA1* gene (24) was amplified by polymerase chain reaction from yeast genomic DNA 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 (Pharmacia Biotech Inc.) and thereby fused with the glutathione *S*-transferase gene (GST). The plasmid was introduced into *E. coli* strain BLR(DE3). For purification of the recombinant RanGAP, cells were grown in 2 liters of LB medium containing 200 μg/ml ampicillin at 30 °C to a cell density of 0.5 A_{600} units. Isopropyl-1-thio-β-D-galactopyranoside was added to 0.2 mM, and cells were grown for 3 h. Cells were harvested by centrifugation at 2,000 × *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 lysed using a French pressure cell, and the lysate was centrifuged at 10,000 × *g* for 10 min. The supernatant was applied to a 1-ml glutathione-Sepharose 4B column (Pharmacia Biotech Inc.) equilibrated in PBS at 4 °C. After washing with 100 ml of PBS, the GST-fusion protein was cleaved on the column by incubation at 21 °C for 12 h with 10 NIH units of thrombin. 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.

Purification of Karyopherin β (Kap95)—Recombinant Kap95 was purified as described previously (19).

Labeling of Ran with [γ-^{32}P]GTP—5 μM Ran was incubated for 30 min on ice with 30 nM [γ-^{32}P]GTP (6000 Ci/mmol, DuPont NEN) in exchange buffer containing 5 mM EDTA, 20 mM Hepes, pH 7.3, and 100 mM KOAc 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 Sephadex G-50 (Pharmacia Biotech Inc.) column prepared in a 1-ml syringe equilibrated with hydrolysis buffer (20 mM Hepes, pH 7.3, 100 mM KOAc, 20 mM MgOAc, 1 mM DTT, and 0.5 mg/ml bovine serum albumin). 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.

GAP Assay—To measure GAP activity, 40 nM Ran-[γ-^{32}P]GTP was incubated either in the absence or presence of various amounts of RanGAP in hydrolysis buffer in a final volume of 100 μl at 21 °C for 10 min. After incubation, 20-μl aliquots were diluted into 1 ml of ice-cold hydrolysis buffer and filtered through nitrocellulose (Schleicher and Schuell). Filters were washed with 5 ml of hydrolysis buffer and dried, and radioactivity was counted in the presence of 3 ml of scintillation fluid (Ready Safe, Beckman) on a Wallac 1410 scintillation counter.

Determination of Complex Formation between Kap95 and Ran-GTP or Ran-GDP—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 Kap95 and either 5 μM Ran-GTP or Ran-GDP was 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 Superdex 200 column (HR30/10; Pharmacia Biotech Inc.) equilibrated with buffer A. Fractions of 0.5 ml were collected, precipitated with 10% trichloroacetic acid, and subjected to SDS-PAGE followed by staining of the gel with Coomassie Blue.

In a control experiment, Ran was first labeled with either [^{3}H]GDP or [γ-^{32}P]GTP. For this, 10 μM Ran was incubated in the presence of either 10 nM [^{3}H]GDP (34 Ci/mmol, DuPont NEN) or 10 nM [γ-^{32}P]GTP (6000 Ci/mmol, DuPont NEN) in exchange buffer for 30 min at 21 °C. This resulted in labeled Ran that contained 16% GTP and 20% GDP, as determined on the reversed-phase column. After addition of 20 mM $Mg(OAc)_2$, 5 μM Ran-[^{3}H]GDP or 5 μM Ran-[γ-^{32}P]GTP was incubated with 2.5 μM Kap95 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.

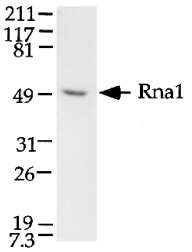

FIG. 1. **Purified yeast RanGAP (Rna1).** RanGAP was purified as a GST-fusion protein as described under "Experimental Procedures." The thrombin-cleaved RanGAP is >90% pure as judged by SDS-PAGE.

Protein Concentrations—These were determined by the method of Bradford (25) using bovine serum albumin as a standard.

RESULTS

Ran (Gsp1), RanGAP (Rna1), and karyopherin β (Kap95), 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 (see Fig. 1 for purified RanGAP and Fig. 4 for purified Ran and karyopherin β). The purified recombinant Ran was determined to contain 40% GDP and 25% GTP, whereas 35% is nucleotide-free (data 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"). Under the conditions used for the exchange reaction (no exogenously added cold GTP), we found that only about 13% of the Ran was complexed with GTP, as determined by measuring the bound nucleotide on a reversed-phase column. Hence, of the 300 nM Ran used in the incubation reaction with RanGAP, only about 40 nM was present as Ran-GTP. After incubation with RanGAP, GTP hydrolysis was measured using a nitrocellulose filter binding assay in which hydrolyzed $^{32}PO_4$ is not retained by the filter, whereas Ran-bound nonhydrolyzed [γ-^{32}P]GTP is retained. The recombinant GAP was indeed active. Increasing concentrations yielded increasing rates of GTP hydrolysis, with as little as 1.0 nM GAP yielding more than 70% GTP hydrolysis and 2.0 nM GAP resulting in near 100% GTP hydrolysis within the 10-min reaction time (Fig. 2). Strikingly, in the presence of 40 nM karyopherin β, the GAP-stimulated GTP hydrolysis was completely inhibited (Fig. 2).

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

To assay directly for an association of Ran with karyopherin β, 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 ensure that most of the Ran would be bound either to GTP or GDP. To allow for complex formation, 2.5 μM karyopherin β was incubated either with 5.0 μM Ran-GTP or Ran-GDP. In a control reaction, 2.5 μM karyopherin β was incubated without Ran-GTP or Ran-GDP. Each of the reaction mixtures was then subjected to gel filtration on a Superdex 200 FPLC column, and fractions were collected, trichloroacetic acid-precipitated, and analyzed by SDS-PAGE and Coomassie blue staining of the gel. The bulk of karyopherin β eluted at fractions 12 and 13 (Fig.

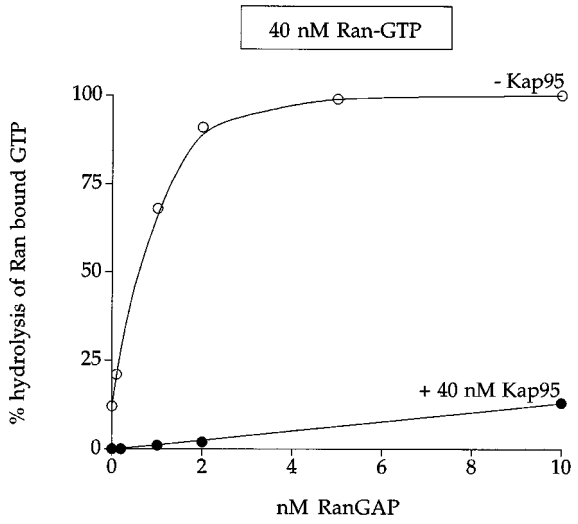

FIG. 2. **GAP activity is inhibited by karyopherin β (Kap95).** GAP activity was assayed as described under "Experimental Procedures" by incubating 40 nM Ran-[γ-^{32}P]GTP in the presence of increasing amounts of RanGAP with (●) or without (○) 40 nM Kap95.

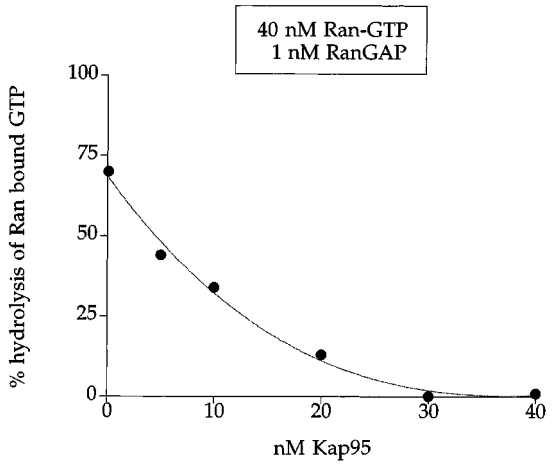

FIG. 3. **Inhibition of GAP activity is dependent on karyopherin β (Kap95) concentration.** GAP activity was determined as described under "Experimental Procedures" after incubating 40 nM Ran-[γ-^{32}P]GTP in the presence of 1 nM RanGAP and increasing amounts of Kap95.

4*A*, *upper panel*). When preincubated with a 2-fold molar excess of Ran-GTP, there was a dramatic shift of the karyopherin β peak to fractions 10 and 11. Moreover, karyopherin β coeluted with about half of the Ran-GTP, whereas the other half of Ran-GTP peaked at fraction 18 (Fig. 4*A*, *middle panel*). These data indicated that karyopherin β and Ran-GTP formed a stoichiometric complex. Some binding to karyopherin β 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 Ran-GDP to karyopherin β, Ran was labeled with [^{3}H]GDP prior to incubation with karyopherin β. Gel filtration on the Superdex 200 column resulted in cofractionation of some of the radioactivity with the karyopherin β-Ran complex (Fig. 4*B*, *open circles*). When Ran was labeled with [γ-^{32}P]GTP prior to incubation with karyopherin β, all of the labeled Ran cofractionated with the karyopherin β-Ran complex (Fig. 4*B*, *closed circles*). These results indicated that Ran-GDP also binds to karyopherin β, but with much lower affinity than does Ran-GTP.

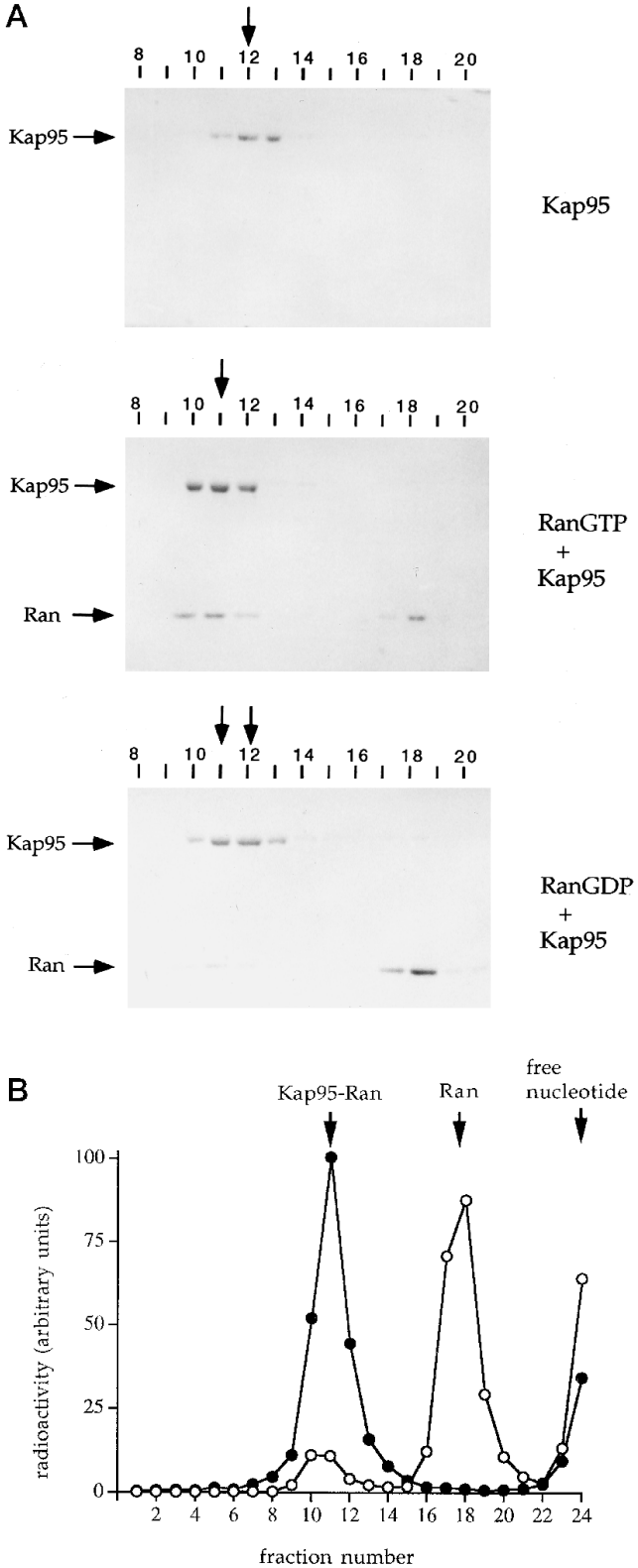

FIG. 4. **Ran forms a complex with karyopherin β (Kap95) that can be detected by gel filtration.** Complex formation between Ran and Kap95 was analyzed on a Superdex 200 FPLC column as described under "Experimental Procedures." 13 of 24 fractions were analyzed by SDS-PAGE. *A*, fractions were analyzed by electrophoresis on a 12% polyacrylamide gel and stained with Coomassie Blue. *Upper panel*, 2.5 μM Kap95 was incubated in buffer A. *Middle panel*, 2.5 μM Kap95 was incubated with 5 μM Ran-GTP. *Lower panel*, 2.5 μM Kap95 was incubated with 5 μM Ran-GDP. *B*, 2.5 μM Kap95 was incubated with 5 μM Ran-[^{3}H]GDP (*open circles*) or 5 μM Ran-[γ-^{32}P]GTP (*closed circles*). Elution of radioactivity was monitored by counting 10-μl aliquots of each of the 24 fractions in a scintillation counter.

DISCUSSION

Our data here show that the RanGAP-stimulated GTP hydrolysis of Ran-GTP is inhibited by karyopherin β in a concentration-dependent manner. Complete inhibition was observed when the amount of karyopherin β was equimolar to Ran-GTP. In gel filtration experiments, Ran-GTP and karyopherin β were found to form a stoichiometric complex. We suggest that binding of Ran-GTP to karyopherin β renders Ran-GTP inaccessible to RanGAP.

Ran-GTP binding to karyopherin β does not appear to affect the slow intrinsic GTPase activity of Ran, as no differences in the rates of GTP hydrolysis could be detected during a 1-h incubation of Ran-GTP either in the absence or presence of karyopherin β (data not shown).

Unlike Ras, which has an affinity for GTP that is about 1 order of magnitude higher than that for GDP (26), Ran has a 10-fold higher affinity for GDP than it has for GTP (27). Up to 80% of the cellular Ran is thought to be located in the nucleus (28), whereas RanGAP is thought to be located in the cytoplasm (29). Hence, the cytoplasmic RanGAP is likely to keep the cytoplasmic concentration of Ran-GTP very low. This seems logical as cytoplasmic Ran-GTP would be detrimental for nuclear import. It would dissociate the karyopherin heterodimer in the cytoplasm, associate with karyopherin β, and thereby prevent targeting of NLS protein to the nuclear pore complex (20).

The formation of a complex between Ran-GTP and karyopherin β that renders Ran-GTP inaccessible to RanGAP is reminiscent of the formation of a complex of Ras-GTP with its downstream effector Raf kinase that has been proposed to render Ras-GTP inaccessible to RasGAP (30, 31). Ras-Raf interaction is thought to be terminated through intrinsic GTP hydrolysis of Ras-GTP, resulting in the release of Raf kinase (32). Similarly, the intrinsic GTPase activity of Ran could result in a dissociation of Ran from karyopherin β as the affinity of the latter for Ran-GDP is lower than for Ran-GTP (Fig. 4).

It remains to be determined whether (Ran-GTP·karyopherin β) complex formation might serve to down-regulate protein import into the nucleus *in vivo*.

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