The Nuclear Transport Factor Karyopherin \( \beta \) 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 \( \beta \) (Rexach, M., and Blobel, G. (1995) Cell 83, 683–692). We show here, using all recombinant yeast proteins expressed in Escherichia coli, that karyopherin \( \beta \) 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 \( \beta \) was dependent on karyopherin \( \beta \) concentration relative to Ran-GTP. Complete inhibition of RanGAP was observed at karyopherin \( \beta \) concentrations that were equimolar to Ran-GTP. In gel filtration experiments, we found Ran-GTP and karyopherin \( \beta \) to form a stoichiometric complex. Ran-GDP bound only weakly to karyopherin \( \beta \). We propose that stoichiometric complex formation between karyopherin \( \beta \) and Ran-GTP renders Ran-GTP inaccessible to RanGAP.

Several soluble transport factors are required for import of a nuclear localization sequence (NLS)-containing protein into the nucleus 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 \( \alpha \) is synonymous with NLS receptor (4), importin (5, 6), importin 60 (7), or importin \( \alpha \) (8); karyopherin \( \beta \) (1) is synonymous with p97 (4, 9), importin 90 (7), or importin \( \beta \) (8). Transport of the NLS protein from the nuclear rim into the nucleus requires the Ran-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 \( \alpha \) and \( \beta \) 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 \( \beta \) 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 \( \beta \) 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 \( \beta \) with Ran-GTP modifies the response of Ran-GTP to RanGAP. Using all recombinant proteins, we found that karyopherin \( \beta \) inhibited RanGAP-stimulated Ran-GTP hydrolysis in a concentration-dependent manner, yielding complete inhibition at karyopherin \( \beta \) concentrations that are stoichiometric to Ran-GTP. In gel filtration experiments, we found that karyopherin \( \beta \) 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 CCT 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 NcoI and BamHI and inserted into NcoI-BamHI-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 ºC to a cell density of 0.6 A600 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 ºC, and the cell pellet was resuspended in 25 ml of ice-cold Tris buffer containing 10 mM Tris-HCl, pH 8.0, 1 mM MgCl2, 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 ºC. 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 MgCl2 for 10 min at 4 ºC. After centrifugation at 10,000 × g, the deaerated 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 Centricron 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 KAc, 2 mM MgOAc, 1 mM EDTA, 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 aliquot injection device and diluted in exchange buffer to a final volume of 100 μl. This solution was subjected to gel filtration on a Superdex 200 FPLC column, and 2 ml aliquots were frozen in liquid nitrogen and stored at −80°C. The bulk of karyopherin Ran was incubated in the presence of 40 mM GDP and 25% GTP, whereas 35% is nucleotide-free (data not shown). To assay for GDP hydrolysis, the endogenous Ran-bound GDP or GTP were in part exchanged with {32P}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 {32P}GTP was not retained by the filter, whereas Ran-bound nonhydrolyzed {32P}GTP is retained. The recombinant GAP was indeed active. Increasing concentrations yielded increasing rates of GDP hydrolysis, with as little as 1.0 mM GAP yielding more than 70% GTP hydrolysis and 2.0 mM 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-{32P}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 GDP or GTP to ensure that most of the Ran would be bound either to GDP or GTP. To allow for complex formation, 2.5 μM karyopherin β was incubated 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.
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 co-eluted with about half of the Ran-GTP, whereas the other half of Ran-GTP peaked at fraction 18 (Fig. 4A, 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. 4A, 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 [3H]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. 4B, open circles). When Ran was labeled with [γ-32P]GTP prior to incubation with karyopherin β, all of the labeled Ran cofractionated with the karyopherin β-Ran complex (Fig. 4B, closed circles). These results indicated that Ran-GDP also binds to karyopherin β, but with much lower affinity than does Ran-GTP.

**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-32P]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-32P]GTP in the presence of 1 nM RanGAP and increasing amounts of Kap95.

**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-[γ-32P]GDP (open circles) or 5 μM Ran-[γ-32P]GTP (closed circles). Elution of radioactivity was monitored by counting 10-μl aliquots of each of the 24 fractions in a scintillation counter.
Karyopherin β Binding to Ran-GTP Inhibits RanGAP

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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REFERENCES

1. Radu, A., Blobel, G., and Moore, M. S. (1995) Proc. Natl. Acad. Sci. U. S. A. 92, 1769–1773
2. Moroianu, J., Blobel, G., and Radu, A. (1995) Proc. Natl. Acad. Sci. U. S. A. 92, 2008–2011
3. Imamoto, N., Tachibana, T., Matsubae, M., and Yoneda, Y. (1995) J. Biol. Chem. 270, 8559–8565
4. Adam, E. J. H., and Adam, S. A. (1994) J. Cell Biol. 125, 547–555
5. Görlich, D., Prehn, S., Laskey, R. A., and Hartmann, E. (1994) Cell 79, 767–778
6. Imamoto, N., Shimamoto, T., Takaoka, T., Tachibana, T., Kose, S., Matsubae, M., Sekimoto, T., Shimonishi, Y., and Yoneda, Y. (1995) EMBO J. 14, 3617–3626
7. Görlich, D., Kostka, S., Kraft, R., Dingwall, C., Laskey, R., Hartmann, E., and Prehn, S. (1995) Curr. Biol. 5, 383–392
8. Görlich, D., Vogel, F., Mills, A. D., Hartmann, E., and Laskey, R. A. (1995) Nature 377, 246–248
9. Chi, N. C., Adam, E. J. H., and Adam, S. A. (1995) J. Cell Biol. 130, 265–274
10. Moore, M. S., and Blobel, G. (1993) Nature 365, 661–663
11. Melchior, F. B., Paschal, J., Evans, J., and Gerace, L. (1993) J. Cell Biol. 123, 1649–1659
12. Moore, M. S., and Blobel, G. (1994) Proc. Natl. Acad. Sci. U. S. A. 91, 10212–10216
13. Paschal, B. M., and Gerace, L. (1995) J. Cell Biol. 129, 925–937
14. Moroianu, J., Hijikata, M., Blobel, G., and Radu, A. (1995) Proc. Natl. Acad. Sci. U. S. A. 92, 6532–6536
15. Nishimoto, T., Ellen, E., and Basilio, C. (1978) Cell 15, 475–483
16. Bischoff, F. R., and Ponstingl, H. (1991) Nature 354, 80–82
17. Bischoff, F. R., Klebe, C., Kretschmer, J., Wittinghofer, A., and Ponstingl, H. (1994) Proc. Natl. Acad. Sci. U. S. A. 91, 2587–2591
18. Coutavas, E., Ren, M., Oppenheim, J. D., D’Eustachio, P., and Rush, M. G. (1993) Nature 366, 585–587
19. Enenkel, C., Blobel, G., and Rexach, M. (1995) J. Biol. Chem. 270, 16495–16502
20. Rexach, M., and Blobel, G. (1995) Cell 83, 683–692
21. Bischoff, F. R., Kreber, H., Kempf, T., Hermes, I., and Ponstingl, H. (1995) Proc. Natl. Acad. Sci. U. S. A. 92, 1749–1753
22. Becker, J., Melchior, F. B., Gerke, V., Bischoff, F. R., Ponstingl, H., and Wittinghofer, A. (1995) J. Biol. Chem. 270, 11860–11865
23. Belhumeur, P., Lee, A., Tam, R., DiPaolo, T., Fortin, N., and Clark, M. W. (1993) Mol. Cell. Biol. 13, 2152–2161
24. Traglia, H. M., Atkinson, N. S., and Hopper, A. K. (1989) Mol. Cell. Biol. 9, 2989–2999
25. Bradford, M. M. (1976) Anal. Biochem. 72, 248–254
26. Neal, S. E., Eccleston, J. F., Hall, A., and Webb, M. R. (1988) J. Biol. Chem. 263, 19718–19722
27. Klebe, C., Bischoff, F. R., Ponstingl, H., and Wittinghofer, A. (1995) Biochemistry 34, 639–647
28. Ren, M., Drivas, G., D’Eustachio, P., and Rush, M. G. (1993) J. Cell Biol. 120, 313–323
29. Hopper, A. K., Traglia, H. M., and Dunst, R. W. (1990) J. Cell Biol. 111, 309–321
30. Warne, P. H., Vidana, P. R., and Downward, J. (1993) Nature 364, 352–355
31. Moodie, S. A., Willumsen, B. M., Weber, M. J., and Wolfman, A. (1993) Science 260, 1658–1660
32. Herrmann, C., Martin, G. A., and Wittinghofer, A. (1995) J. Biol. Chem. 270, 2901–2905