Ran-binding Protein 3 Links Crm1 to the Ran Guanine Nucleotide Exchange Factor

Received for publication, October 26, 2001, and in revised form, March 27, 2002 Published, JBC Papers in Press, April 3, 2002, DOI 10.1074/jbc.C100620200

Michael E. Nemergut‡, Mark E. Lindsay‡, Amy M. Brownawell¶, and Ian G. Macara§

From the Departments of ‡Microbiology and ¶Pharmacology, The Center for Cell Signaling, The University of Virginia, Charlottesville, Virginia 22908

Ran-binding protein 3 (RanBP3) is an ~55-kDa protein that functions as a cofactor for Crm1-mediated nuclear export. RanBP3 stimulates export by enhancing the affinity of Crm1 for Ran-GTP and cargo. However, important additional functions for this cofactor may exist. We now report that RanBP3 associates with the Ran-specific guanine nucleotide exchange factor, regulator of chromosome condensation 1 (RCC1). This interaction was stimulated by the addition of Ran; moreover, Ran-GDP, Ran-GTP, and Ran without nucleotide could all stimulate complex formation between RanBP3 and RCC1 even though binding of Ran-GDP to RanBP3 alone was undetectable. RanBP3 could also promote binding of Crm1 to RCC1 in the presence of Ran. Binding of RanBP3 to RCC1 increased the catalytic activity of RCC1 toward Ran, and importantly, the ability of RanBP3 to stimulate RCC1 was not affected by the presence of Crm1. These data indicate that RanBP3 acts as a scaffold protein to promote the efficient assembly of export complexes. By tethering Crm1 to catalytically enhanced RCC1, RanBP3 may lower the entropic barrier for the loading of Ran-GTP onto Crm1. We propose that this provides an additional mechanism by which RanBP3 facilitates export.

Nuclear transport is an essential activity in all eukaryotic cells (1, 2). All nuclear transport is believed to occur through the nuclear pore complex (NPC), a large glycoprotein complex that spans the double membrane of the nuclear envelope (3). The NPC contains a central channel that permits transit of cargoes between the nucleus and cytoplasm. The NPC is composed of proteins called nucleoporins that possess binding sites for nuclear transport receptors. These receptors (called importins, exportins, transportins, and karyopherins) are able to bind simultaneously to their cargo and nucleoporins to allow transport from one compartment to the other.

The Ran GTPase controls most vectorial transport between the nucleus and cytoplasm. Like other GTPases, Ran cycles between GDP- and GTP-bound states. A single guanine nucleotide exchange factor (RCC1) and GTPase-activating protein (RanGAP) catalyze the Ran GDP/GTP cycle (4, 5). These proteins possess distinct subcellular localizations. While RanGAP is localized to the cytoplasm and NPC (6), RCC1 is a nuclear protein tethered to chromatin through histones H2A/H2B (6–8). The disparate subcellular localizations of RanGAP and RCC1 predict a steep gradient of Ran-GTP across the nuclear envelope, which is crucial for most forms of nuclear transport.

A defining characteristic of β-karyopherin transport receptors is their direct interaction with Ran-GTP. The effect of Ran on the interaction between the receptor and its cargo is dependent upon the nature of the transport receptor. Ran-GTP dissociates cargo from import receptors, while in contrast, Ran-GTP is required for an export receptor to bind its cargo (1, 2). Upon transit of an export complex into the cytoplasm, hydrolysis of Ran-GTP to Ran-GDP causes release of the cargo from the export receptor. Thus, one essential function of Ran is to regulate the interactions between receptors and cargo in a compartment-specific manner.

The best characterized nuclear export receptor is Crm1 (also called exportin-1 or Xpo1p in Saccharomyces cerevisiae). Crm1 is able to associate with Ran-GTP and with cargo proteins harboring a leucine-rich nuclear export signal (NES) (9–12). The Crm1-cargo-Ran-GTP complex translocates across the NPC and is then dissociated by hydrolysis of GTP on Ran, stimulated by RanGAP and other required factors. Recently several cofactors have been identified that enhance nuclear export by Crm1. Nxt1 binds directly to Crm1 and Ran and facilitates the delivery of the export complex to the cytoplasmic face of the NPC (13). Another protein, eukaryotic initiation factor-5A, may enhance the affinity of Crm1 for the NES of the human immunodeficiency virus, type 1 protein Rev (14). Most recently Ran-binding protein 3 (RanBP3) has been shown to enhance nuclear export mediated by Crm1 (15, 16). RanBP3 binds directly to Crm1, and the Crm1-RanBP3 complex has an enhanced affinity for both Ran-GTP and cargo. The ternary Crm1-RanBP3-Ran-GTP-NES complex is able to interact with the NPC, mediating transport from the nucleus to the cytoplasm. In the absence of Ran-GTP, RanBP3 prevents Crm1 from binding the NPC, thereby preventing futile export events.

Here we describe an additional independent mechanism by which RanBP3 may enhance nuclear export by Crm1. We demonstrate that RanBP3 can interact with the Ran exchange factor RCC1 in a Ran-stimulated manner. This interaction increases the catalytic activity of RCC1, accelerating nucleotide exchange by ~10-fold. The activation of RCC1 by RanBP3 can be amplified by histones H2A/H2B, suggesting that RanBP3 can stimulate RCC1 at the chromatin surface. Furthermore, RanBP3 can promote binding of Crm1 to RCC1, thereby tethering the export receptor to a region of accelerated nucleotide exchange. We propose that RanBP3 acts as a scaffold protein to promote the efficient assembly of Crm1-dependent export complexes.
Experimental Procedures

Cloning, Protein Expression, and Purification—All glutathione S-transferase (GST) fusion proteins were prepared as described previously (17). Histones H2A and H2B were purchased from Roche Molecular Biochemicals. RCC1 and RCC1(D182A) were cloned into a bait plasmid containing the DNA-binding domain of Gal4p as an ~120-bp BamHI/BamHI fragment. RCC1 was also cloned into a BamHI/HindIII fragment into pHis-Parallel2 and expressed in Escherichia coli (BL21) by induction with 0.4 mM isopropyl-1-thio-β-D-galactopyranoside at 18 °C for 16 h. After lysis of the bacteria, recombinant RCC1 was purified on Ni3+-agarose (Qiagen). The His6 tag was removed from RCC1 by incubation with the tobacco etch virus protease (Invitrogen). Both the cleaved His6 tag and tobacco etch virus protease were removed on Ni3+-agarose (Qiagen). RanBP3, Ran, GST-R, GST-NF, and Crm1 were prepared as described previously (15).

Yeast Two-hybrid Analysis—Yeast dihybrid mating assays were performed as described previously (15, 18). Bait plasmids encoding RCC1 or RCC1(D182A) fused to the DNA-binding domain of GAL4 were transformed into HF7c (MATa). Prey plasmids encoding RanBP3 fused to the transcriptional activation domain of VP16 were transformed into W303 (MATα). The strains were mated and plated on Leu-Trp-His+ medium. Exchange Assays—RCC1-catalyzed exchange assays were performed as described previously (7, 19). Briefly, recombinant Ran was loaded with [α-32P]GDP or [α-32P]GTP by incubation in 25 mM MOPS (pH 7.1) and 1 mM EDTA. After incubation on ice for 20 min, the reaction was quenched by the addition of 10 mM MgCl2. All exchange assays were performed at 30 °C for 3 min in exchange assay buffer (25 mM MOPS (pH 7.1), 7.5 mM MgCl2, 0.6 mM NaH2PO4, 0.5 mg/ml BSA, and 1 mM GDP or GTP as indicated) containing ~0.6 mM RCC1. Reactions were stopped by binding to nitrocellulose filters using a vacuum apparatus. After washing the filters with ice-cold buffer (7.5 mM NaH2PO4 (pH 6.8), 7.5 mM NaH2PO4 (pH 6.8), 10 mM MgCl2, and 1 mM ATP), the reactions were analyzed by scintillation counting. Dissociation rate constants (koff) were calculated assuming a single exponential decay (ln(Ct/C0) = -koff+t, where C0 is the radioactive counts at time 0 and Ct is the counts at time t).

Immunoblotting—RCC1 was visualized using an anti-RCC1 antibody (1:200; N-19, Santa Cruz Biotechnology) and a horseradish peroxidase-coupled rabbit anti-goat secondary antibody (1:20,000; The Jackson Laboratories). Antibody (1:2,000; Transduction Laboratories) and a horseradish peroxidase-coupled rabbit anti-goat secondary antibody (1:20,000; The Jackson Laboratories).

Binding Assays—GST fusion proteins were immobilized on glutathione (GSH)-Sepharose (Pharmacia) in phosphate-buffered saline. After blocking the beads for 1 h at 4 °C in 5% BSA, phosphate-buffered saline (w/v), binding reactions were accomplished in binding buffer (20 mM HEPES (pH 7.4), 150 mM NaCl, 2 mM MgCl2, 1 mg/ml BSA, 0.1% Tween 20 (v/v), 14 mM 2-mercaptoethanol, and 1 mM GDP or GTP). Wild-type Ran was treated with immobilized GST-RanGAP for 90 min at 25 °C prior to use in binding assays to ensure that all Ran protein was GDP-bound. For all binding reactions, the indicated form of Ran (10 μM), RanBP3 (10 μM), RCC1 (10 μM), or Crm1 (1 μM) was added. For all assays using Crm1, the binding buffer was modified by increasing the NaCl and 2-mercaptoethanol to 300 and 28 mM, respectively. Beads were then washed in binding buffer without BSA. One-half of the total bound proteins and 1/20 of the total unbound proteins were analyzed by SDS-PAGE and immunoblotting.

Results

RanBP3 has been shown to stimulate Crm1-dependent nuclear export of NES-bearing cargoes in part by increasing the affinity of Crm1 for its substrate (15, 16). However, it was unclear whether RanBP3 has additional distinct functions in nuclear transport. RanBP3 was originally cloned from a yeast two-hybrid screen using RCC1 as bait (20), which suggested that RanBP3 might play a role in regulating nucleotide exchange on Ran. We first confirmed this interaction using a yeast dihybrid mating assay. As shown in Fig. 1A, RCC1 was able to support growth in a RanBP3-dependent manner. These data suggest that RCC1 and RanBP3 are able to interact. However, RCC1(D182A), a mutant that is unable to interact with Ran and catalyze nucleotide exchange (21), did not support growth with RanBP3. Therefore, the RCC1-RanBP3 interaction appears to be dependent upon the ability of RCC1 to interact with Ran. These data are in agreement with those reported previously for RanBP3 (20) and the S. cerevisiae homologue Yrb2p (22).

To determine whether Ran acts as a bridge to link RCC1 and RanBP3, we first analyzed the ability of RanBP3 to interact with Ran. RCC1 can interact with both Ran-GDP and Ran-GTP (4, 23); in contrast, RanBP3 has been reported to bind specifically, although with low affinity, only to Ran-GTP (20). To test these interactions, GST-tagged RanBP3 (GST-RanBP3) or GST alone was immobilized to GSH beads and incubated with Ran(G69L) (a mutant Ran locked in the GDP-bound state (all 10 μM), was incubated with GST or GST-RanBP3 bound to GSH beads. Bound and unbound proteins were subjected to SDS-PAGE and immunoblotting. C, RCC1 was incubated ± Ran(G69L) or wild-type Ran-GDP with GST or GST-BP3 bound to GSH beads. Samples were analyzed as above. D, RCC1 ± Ran(G69L) was incubated with GST, GST-BP3, GST-R (RanBP3 RBD), or GST-NF (RanBP3 minus the RBD) bound to beads. Samples were analyzed as above.
RanBP3 contains other domains in addition to the Ran-binding domain (RBD) (15). To determine which region of RanBP3 is responsible for the formation of the trimeric complex, we tested two fragments of the protein for binding to RCC1. The isolated RBD (GST-R) bound RCC1 in the presence of Ran-GTP, while a fragment that lacks the RBD (GST-NF) did not bind RCC1 (Fig. 1D). Therefore, the RanBP3 RBD is both necessary and sufficient to mediate the interaction between RanBP3, Ran, and RCC1.

Because RanBP3 can bind several of the putative reaction intermediates of RCC1-catalyzed nucleotide exchange (23), we asked whether RanBP3 affects the enzymatic activity of RCC1. Ran, preloaded with [α-32P]GDP or [α-32P]GTP, was incubated with a fixed amount of RCC1 in the presence or absence of RanBP3. As shown in Fig. 2, RanBP3 increased the catalytic activity of RCC1. Ran, preloaded with [α-32P]GDP or [α-32P]GTP, was incubated with a fixed amount of RCC1 in the presence or absence of RanBP3. As shown in Fig. 2, RanBP3 increased the catalytic activity of RCC1. RanBP3 increased the dissociation rate constant (koff) for both [α-32P]GDP and [α-32P]GTP, although the effect for GDP appeared more robust. Furthermore, RanBP3 enhanced exchange of [α-32P]GDP for unlabeled GDP (data not shown). Because RanBP3 cannot bind directly to Ran-GDP, its nucleotide state. Hence, of RCC1, RanBP3 is able to recognize Ran irrespective of its nucleotide state.

RanBP3 has been shown to enhance Crm1-dependent nuclear export (15, 16). RanBP3 binds directly to Crm1 and enhances the affinity of Crm1 for both Ran-GTP and cargo. Therefore, since RCC1 can bind RanBP3, it seemed possible that RCC1 might also interact with Crm1 in a RanBP3-dependent manner. To test this hypothesis directly, GST-RCC1 was immobilized to beads and incubated with Crm1 in the presence or absence of Ran and RanBP3. In the absence of these proteins, only background levels of Crm1 were bound to RCC1 (Fig. 3A). Ran bound RCC1; however, it did not facilitate binding of Crm1. RanBP3 associated with RCC1 in a Ran-stimulated manner (Fig. 3A). Only when Crm1, RanBP3, and Ran were added could Crm1 associate with RCC1 (Fig. 3A). Thus, Crm1 is able to bind RCC1 in a Ran-stimulated and RanBP3-dependent manner, indicating that RCC1, Ran, RanBP3, and Crm1 are able to form a quaternary complex. Interestingly, although...
both Ran-GTP and Ran-GDP were able to stimulate binding of Crm1 to RCC1 in the presence of RanBP3. Ran-GDP appeared more efficient at recruiting Crm1 to RCC1 (Fig. 3B). These data suggest that the Crm1-RanBP3 complex can more efficiently recognize Ran-GDP bound to RCC1 than Ran-GTP. Importantly, when added to exchange assays, Crm1 did not alter the ability of RanBP3 to stimulate RCC1 (Fig. 3C).

**DISCUSSION**

RanBP3 is a cofactor for Crm1-dependent nuclear export (15) and functions by increasing the affinity of Crm1 for both Ran-GTP and cargo (15). Although the binary Crm1-RanBP3 complex is able to bind both Ran-GTP and cargo onto Crm1, RanBP3 functions by binding directly to Crm1. Although the binary Crm1-RanBP3 complex is able to bind both Ran-GTP and cargo (15), it has been predicted that ~80% of free nuclear Ran is GDP-bound (32) and cannot facilitate binding to the Crm1-RanBP3-Ran-GTP complex. RanBP3, which functions by increasing the affinity of Crm1 for both Ran-GTP and Ran-GDP, is able to facilitate this process. Furthermore, in the presence of the RanBP3-Crm1 complex, the enzymatic activity of RCC1 toward Ran is stimulated.

Based on these data, we propose that nuclear RanBP3 acts as a scaffold protein to facilitate efficient loading of Ran-GTP and cargo onto Crm1. RanBP3 functions by binding directly to Crm1. Although the binary Crm1-RanBP3 complex is able to bind both Ran-GTP and cargo onto Crm1, RanBP3 functions by binding directly to Crm1. Although the binary Crm1-RanBP3 complex is able to bind both Ran-GTP and cargo (15), it has been predicted that ~80% of free nuclear Ran is GDP-bound (32) and cannot facilitate binding of Crm1-RanBP3 to cargo. In contrast, Ran-GDP can recruit RanBP3 to Crm1. This recruitment stimulates the catalytic activity of RCC1, thereby promoting rapid loading of GTP. Conversion of Ran-GDP to Ran-GTP within the RanBP3-Ran-GTP-RCC1 complex would promote export cargo binding to the Crm1. Release of the Crm1-RanBP3-Ran-GTP-export cargo complex from RCC1 may require additional cellular factors since in vitro binding of cargo is not sufficient to promote disassembly (data not shown). Alternatively, no allosteric release mechanism may be necessary if the dissociation rate constant is sufficiently high.

Although neither Crm1, RanBP3, nor the Crm1-RanBP3 complex can detectably bind Ran-GDP (Fig. 1B, data not shown), both RanBP3 and Crm1-RanBP3 can be recruited to RCC1 by Ran-GDP (Figs. 1 and 3). These data demonstrate that RCC1 can remodel the structure of Ran-GDP such that it can be recognized by the Crm1-RanBP3 complex. Also, although RanBP3 possesses a RBD similar to that of RanBP1, RanBP3 possesses a low affinity for Ran-GTP relative to RanBP1, and while RanBP3 stimulates nucleotide exchange by RCC1, RanBP1 is inhibitory (Fig. 1C) (24). However, it has been noted that critical residues for Ran binding within the RBD from RanBP1 are not present in RanBP3 (25). The residues involved in the GTP/GDP switch may provide the basis for the Ran-GTP-specific binding of RanBP1. Their absence in RanBP3 may account for the ability of RanBP3 to bind Ran-GDP in the presence of RCC1. We note that Nup2p, another protein with a RanBP1-like RBD, also does not inhibit RCC1 (26). Together these data demonstrate that, while the RBDs from RanBP1 and RanBP3 are similar, they harbor distinct biochemical activities. Furthermore, they demonstrate that the inhibitory activity of RanBP1 on RCC1 is not intrinsic to all proteins possessing a RanBP1-like RBD (see below).

Solution of the three-dimensional crystal structure of the RCC1-Ran complex has provided insight into the enzymatic mechanism of RCC1 (27). One interesting aspect of the structure is that the C terminus of Ran appears to be deployed, suggesting that RCC1 can activate this conformational switch in Ran. This hypothesis is supported by the fact that RCC1 can enhance nucleotide exchange on a mutant Ran lacking its C-terminal acidic tail (Ran$^{211\text{DEDDDL}}$) more rapidly than on wild-type Ran (19). We suspected that RanBP3 might stimulate exchange by facilitating the C-terminal switch. However, this mechanism is unlikely because RanBP3 is capable of stimulating RCC1-mediated exchange even on Ran$^{211\text{DEDDDL}}$ (data not shown).

Although RCC1 is an essential protein for nuclear transport, it is also essential for nuclear envelope assembly and mitotic spindle formation (28–30). By binding to chromatin, it is believed that RCC1 creates a high concentration of Ran-GTP at the chromatin surface. Interestingly, binding of RCC1 to histones H2A/H2B enhances the catalytic activity of RCC1, and RanBP3 can further amplify the effect of histones. Thus, RanBP3 may possess a role in cell cycle progression and nuclear envelope biogenesis. The yeast homologue of RanBP3, Yrb2p, has been implicated in cell cycle progression since disruption of YRB2 results in prolongation of mitosis (31). Thus, a potential role for RanBP3 in the mammalian cell cycle is worthy of future research.

**Acknowledgments**—We thank L. Gerace for providing the anti-Ran serum. D. Gorchil, for the anti-Ran(RQ89L) cDNA, and T. Nishimoto for the RCC1 cDNA. We thank all members of the Macara laboratory for help during all phases of this study.

**REFERENCES**

1. Gorchil, D., and Kutay, U. (1999) *Annu. Rev. Cell Dev. Biol.* 15, 667–660
2. Nakanishi, S., and Dreyfuss, G. (1999) *Cell* 96, 677–690
3. Stoffler, D., Fransen, P., and Aebi, U. (1999) *Curr. Opin. Cell Biol.* 11, 391–401
4. Bischoff, F. R., and Ponstingl, H. (1991) *Nature* 354, 80–82
5. Bischoff, F. R., Klebe, C., Kreth, J., Witthoff, A., and Ponstingl, H. (1994) *Proc. Natl. Acad. Sci. U. S. A.* 91, 2587–2591
6. Hopper, A. K., Traglina, H. M., and Dunst, R. W. (1990) *J. Cell Biol.* 111, 309–321
7. Nemerget, M. E., Mizzen, C. A., Stuckenberg, T., Allen, C. D., and Macara, I. G. (2001) *Science* 292, 1540–1543
8. Ohkubo, M., Okazaki, H., and Nishimoto, T. (1989) *J. Cell Biol.* 109, 1389–1397
9. Fornerod, M., Ohno, M., Yoshida, M., and Mattaj, I. W. (1997) *Cell* 90, 1051–1060
10. Fukuda, M., Asano, S., Nakamura, T., Adachi, M., Yoshida, M., Yanagida, M., and Nishida, E. (1997) *Nature* 390, 308–311
11. Ossareh-Nazari, B., Bachelier, F., and Dargemont, C. (1997) *Science* 278, 141–144
12. Stade, K., Ford, C. S., Guthrie, C., and Weis, K. (1997) *Cell* 90, 1041–1050
13. Black, B. E., Holaska, J. M., Levesque, L., Ossareh-Nazari, B., Gwizdek, C., Dargemont, C., and Paschal, B. M. (2001) *J. Cell Biol.* 152, 141–155
14. Hofmann, W., Reinhart, B., Ewald, A., Muller, E., Schmitt, I., Staubner, R. B., Lottspeich, F., Jekisch, B. M., Scheer, U., Hauber, J., and Dahaubale, M. C. (2001) *J. Cell Biol.* 152, 895–910
15. Lindsay, M. E., Holaska, J. M., Welch, K., Paschal, B. M., and Macara, I. G. (2001) *J. Cell Biol.* 153, 1391–1402
16. Englmeier, L., Fornerod, M., Bischoff, F. R., Petosa, C., Mattaj, I. W., and Kutay, U. (2001) *EMBO Rep.* 2, 926–932
17. Nemerget, M. E., and Macara, I. G. (2000) *J. Cell Biol.* 149, 835–850
18. Pfaffer, S. M., and Macara, I. G. (1999) *EMBO J.* 18, 5502–5513
19. Richards, S. A., Lounsbury, K. M., and Macara, J. I. (1995) *J. Biol. Chem.* 270, 14405–14411
20. Mueller, L., Cordes, V. C., Bischoff, F. R., and Ponstingl, H. (1998) *FEBS Lett.* 427, 330–336
21. Azuma, Y., Seino, H., Seki, T., Uzawa, S., Klebe, C., Ohba, T., Witthoff, A., Hayashi, N., and Nishimoto, T. (1996) *J. Biochem. (Tokyo)* 120, 82–91
22. Taura, T., Schlenstedt, G., and Silver, P. A. (1997) *J. Biol. Chem.* 272, 31877–31884
23. Klebe, C., Prinz, H., Witthoff, A., and Goody, R. S. (1995) *Biochemistry* 34, 12543–12552
24. Bischoff, F. R., Krebber, H., Smirnova, E., Dong, W., and Ponstingl, H. (1995) *EMBO J.* 14, 705–715
25. Macara, I. G. (1999) *Curr. Biol.* 9, R436–R439
26. Denning, D., Oleyna, G., and Macara, I. G. (2002) *Science* 295, 488–491
Ran-binding Protein 3 Links Crm1 to the Ran Guanine Nucleotide Exchange Factor
Michael E. Nemergut, Mark E. Lindsay, Amy M. Brownwell and Ian G. Macara

J. Biol. Chem. 2002, 277:17385-17388.
doi: 10.1074/jbc.C100620200 originally published online April 3, 2002

Access the most updated version of this article at doi: 10.1074/jbc.C100620200

Alerts:
• When this article is cited
• When a correction for this article is posted

Click here to choose from all of JBC’s e-mail alerts

This article cites 32 references, 16 of which can be accessed free at
http://www.jbc.org/content/277/20/17385.full.html#ref-list-1