Ran-Binding Protein 3 Is a Cofactor for Crm1-mediated Nuclear Protein Export

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Abstract. Crm1 is a member of the karyopherin family of nucleocytoplasmic transport receptors and mediates the export of proteins from the nucleus by forming a ternary complex with cargo and Ran:GTP. This complex translocates through the nuclear pores and dissociates in the cytosol. The yeast protein Yrb2p participates in this pathway and binds Crm1, but its mechanism of action has not been established. We show that the human orthologue of Yrb2p, Ran-binding protein 3 (RanBP3), acts as a cofactor for Crm1-mediated export in a permeabilized cell assay. RanBP3 binds directly to Crm1, and the complex posseses an enhanced affinity for both Ran:GTP and cargo. RanBP3 shuttles between the nucleus and the cytoplasm by a Crm1-dependent mechanism, and the Crm1–RanBP3–NES–Ran:GTP quaternary complex can associate with nucleoporins. We infer that this complex translocates through the nuclear pore to the cytoplasm where it is disassembled by RanBP1 and Ran GTPase–activating protein.

Key words: Crm1 • nuclear export • permeabilized cells • Ran • RanBP3

Introduction

Eukaryotic cells are defined by the presence of a membrane-bounded nucleus that separates transcription from translation. Cells must transport many types of protein and nucleic acids across the nuclear membrane. This cargo moves through the nuclear pore complexes (NPCs), which are macromolecular assemblies that penetrate the nuclear double membrane, (for review see Davis, 1995). The translocation of cargo is often facilitated by soluble receptors that can bind NPCs and also recognize targeting signals within the cargo (Gorlich, 1998; Mattaj and Englmeier, 1998; Pemberton et al., 1998; Nakielny and Dreyfuss, 1999). These receptors have been variously termed karyopherins, importins, exportins, and transportins. Although possessing only limited sequence similarity, they have the common property of binding to nuclear pore components (nucleoporins) and to the small GTPase Ran. Ran is predominantly nuclear at steady state (Bischoff and Ponstingl, 1991). The guanine nucleotide exchange factor for Ran (RanGEF) is confined within the nucleus (Bischoff and Ponstingl, 1991), whereas a Ran GTPase–activating protein (RanGAP) is located both in the cytosol (Ohtsubo et al., 1989; Hopper et al., 1990; Bischoff et al., 1994) and tethered to the external face of the NPC (Matutis et al., 1996; Mahajan et al., 1997; Saitoh et al., 1997). The compartmentalization of these factors is believed to create a steep gradient of Ran:GTP across the NPC, and the asymmetric distribution of Ran:GTP is a major determinant imparting directionality upon nuclear transport (Gorlich, 1998; Mattaj and Englmeier, 1998; Pemberton et al., 1998; Macara et al., 2000). Ran:GTP binds to the karyopherin class of nuclear transport receptors and promotes either the assembly or disassembly of receptor–cargo complexes.

Nuclear protein export cargo is frequently identified by a leucine-rich sequence termed a nuclear export signal (NES). A leucine-rich NES has been identified in the protein kinase A inhibitor (PKI) as well as RanBP1, MAPKK, HIV Rev, and many other proteins (Wen et al., 1995; Fukuda et al., 1996; Richards et al., 1996). The leucine-rich NES is recognized by the karyopherin family member, Crm1 (exportin-1 or Xpo1p in S. cerevisiae) (Fornerod et al., 1997a; Fukuda et al., 1997; Ossareh-Nazari et al., 1997; Stade et al., 1997). Within the nucleus, Crm1 can form a ternary complex with NES-containing cargoes and Ran:GTP and translocate through the NPC. A recently discovered cofactor, Nxt1, facilitates delivery of the

*Abbreviations used in this paper: BHK21, baby hamster kidney; bPKI, biotinylated PKI; GFP, green fluorescent protein; GGRanBP1, GFP–RanBP1 fusion protein; GST, glutathione S-transferase; HA3–RanBP3, triple hemagglutinin–tagged RanBP3; LMB, leptomycin B; NES, nuclear export signal; NLS, nuclear localization signal; NPC, nuclear pore complex; PKI, protein kinase A inhibitor; RanBP3, Ran-binding protein 3; RanGAP, Ran GTPase–activating protein; RBD, Ran-binding domain; STV, streptavidin.
export complex to the cytoplasmic side of the NPC, where the complex docks on the nucleoporin CAN/Nup 214, until it is released by association with RanGAP and RanBP1 (Askjaer et al., 1999; Black et al., 1999, 2001; Katahira et al., 1999; Kehlenbach et al., 1999). These two factors together catalyze the hydrolysis of Ran:GTP to Ran:GDP. Complex disassembly may precede GTP hydrolysis as the latter is not strictly required for in vivo nuclear export over short time periods (Richards et al., 1997). The unloaded Crm1 can presumably shuttle back through the pore to complete the cycle.

Although the current model addresses the vectoriality of nuclear export, it is likely that the actual process is considerably more complex. For instance, the affinities of many endogenous export cargoes for Crm1 are remarkably low even in the presence of Ran:GTP (Askjaer et al., 1999; this study). The affinity of Crm1 for the HIV-1 protein, Rev, is increased substantially by eIF-5A (Hofmann et al., 2001) but a similar effect has not been observed for any endogenous cargo, so it remains unclear whether eIF-5A normally functions as an export cofactor. Nonetheless, we reasoned that additional cofactors may participate in Crm1-mediated nuclear protein export to facilitate endogenous cargo loading.

Here, we show that Ran-binding protein 3 (RanBP3) behaves as a cofactor for Crm1-mediated export. RanBP3 binds to Crm1, and the complex possesses a higher affinity for Ran:GTP and for NES-containing cargo. We present evidence that, after assembly in the nucleus, the Crm1–RanBP3–Ran:GTP–NES cargo complex interacts with the NPC. After translocation through the pore into the cytoplasm, this quaternary complex is disassembled via the concerted action of RanBP1 and RanGAP.

Materials and Methods

DNA and Peptide Constructs

The cDNA encoding full-length RanBP3 was inserted in pGEX-2T1 (Amersham Pharmacia Biotech) and pHK3 vectors (Welch et al., 1999). RanBP3 deletions were created by PCR amplification of the indicated domains and ligation into pGEX-2T1. pQE60-Crm1 was a gift from Iain Mattaj (EMBL, Heidelberg, Germany), pQE30-RanQ69L from D. Görlich (University of Heidelberg, Heidelberg, Germany), and p2 DNA from John Hanover (National Institutes of Health, Bethesda, MD). All constructs were checked by automated sequencing. Peptides used in binding and GAP assays: MVN NS2 NES peptide, CVDEMT-KKFGITLTHDTEK (Askjaer et al., 1999); PKI NES peptide, GSNE-LA-LKLAGLDNKTGGC (Holaska and Paschal, 1998); and as a control peptide, CGAGRSSMAHGPGA.

Protein Expression and Purification

Glutathione S-transferase (GST) fusion proteins were expressed in Escherichia coli XLI-Blue as described previously (Lounsberry and Macara, 1997; Welch et al., 1999). Proteins were bound to glutathione-Sepharose beads (Amersham Pharmacia Biotech), eluted with glutathione, and exchanged into thrombin cleavage buffer (50 mM Tris, pH 7.5, 150 mM NaCl, and 2.5 mM CaCl2) using a PD10 column (Amersham Pharmacia Biotech) (Nemergut and Macara, 2000). Proteins were cleaved with thrombin, and GST and thrombin were removed with glutathione-Sepharose and p-aminobenzamidine beads (Sigma-Aldrich), respectively. His-tagged RanQ69L and Crm1-His, were expressed and purified on Talon beads (CLONTECH Laboratories, Inc.), as described previously (Askjaer et al., 1999). Addition of 28 mM 2-mercaptoethanol was required to insure recovery of functional Crm1 protein. Proteins were concentrated using a Centricron 30 (Amicon).

Protein Binding Assays

GST fusion proteins were bound to glutathione–Sepharose beads in 3% BSA/PBS for 1 h at 4°C. Beads were washed 2× with buffer A (20 mM Hepes, pH 7.4, 150 mM KOAc, 2 mM MgOAc, 0.1% Tween 20, 28 mM β-ME, 0.1% BSA) and then added to solutions containing combinations of transport factors preassembled in this buffer. After incubation with shaking (1 h at 4°C), the beads (10 μl) were washed 4× with 1.0 ml buffer A without BSA, subjected to SDS-PAGE, and analyzed by immunoblotting. Proteins were detected with monoclonal antibodies against the His tag (1:2,000; Babco), GST (gift of T. Parsons, University of Virginia, Charlottesville, VA; 1:10,000), Ran (1:1,800; Transduction Laboratories), Nup214 (RL-1, 1:1,000), p62 (1:1,000; Transduction Laboratories) or polyclonal antibodies against Crm1 (gift of L. Gersae, The Scripps Research Institute, La Jolla, CA; 1:100,000), or polyclonal antibodies against RanBP1 (1:200; Santa Cruz Biotechnology, Inc.), followed by HRP-conjugated secondary antibodies (1:20,000; The Jackson Laboratory) and exposure to a chemiluminescence reagent (KPL). Quantitation of immunoblotting was performed with a densitometer (Molecular Dynamics); and analysis, with ImageQuant v3.3 software (Molecular Dynamics).

Peptides were conjugated to Sulfolink® beads (Pierce Chemical Co.) according to the manufacturer’s instructions. Beads were blocked in 3% BSA/PBS (1 h at 4°C) and then washed 2× with buffer B (20 mM Hepes, pH 7.4, 150 mM NaCl, 2 mM MgOAc, 0.1% Tween 20, 28 mM β-ME, 0.1% BSA) and shaken for 2 h at 4°C with transport factors preassembled in binding buffer. After washing with buffer B (substituted with 300 mM NaCl), the beads were analyzed as described above.

Binding reactions on Ni²⁺-NTA beads (QIAGEN) were performed with buffer C (20 mM Hepes, pH 7.4, 300 mM NaCl, 6 mM MgOAc, 0.1% Tween 20, 14 mM β-ME, 2% BSA, 10 mM imidazole). After loading with Crm1–H4 in buffer C without imidazole, beads were aliquoted and exposed to transport factors for 1 h at 4°C. The beads were then resuspended in buffer C and drawn into preblocked Wizard minicolumns (Promega), and washed 2× with 3 ml of buffer C before scintillation counting.

ELISA assays were performed as described previously (Harlow and Lane, 1999). In brief, Crm1 was immobilized on polystyrene 96-well plates. After blocking with 3% BSA/0.1% Tween 20/PBS, the wells were exposed to dilutions of GST-RanBP3 containing RanQ69L, RanQ69L and NES, or buffer alone, for 1 h in buffer A. After washing, GST was identified with anti-GST monoclonal antibody (1:20,000), followed by anti-mouse HRP-conjugated antibody (1:4,000). After exposure to 3’3’,5’5’-tetramethylbenzidine (Sigma-Aldrich) for 10 min, the solution was acidified with 0.5 M H₂SO₄ and read at 410 nM on a ELX-800 plate reader (Bio-Tek Instruments). Binding data were fit by an iterative procedure to an equation describing a single binding site: fractional saturation, s = x/(K_app + x) (where x = Crm1 in immunoblotting and GST-RanBP3 for ELISA). Analysis was performed using Kaleiderg v3.5 (Synergy Software).

Nucleoporin immunoprecipitations were performed as described (Kehlenbach et al., 1999) and supplemented with Crm1 (50 nM), RanBP3 (200 nM), RanQ69L (2 μM), and/or MVN NS2 NES peptide (4 μM).

Yeast Dihybrid Analysis

Bait plasmids encoding fusion proteins of the DNA-binding domain of Gal4p and human importin 11, CAS, importin-β, or Crm1 were transformed into a HF7c yeast strain. HF7c yeast possess a green fluorescent protein (GFP)–streptavidin–nuclear localization signal (NLS) fusion protein (Black et al., 1999). After 48 h, the cells were washed with HBSS and incubated for 1 h in DME 5% FCS/5% CS plus 35.5 μM cycloheximide. Where indicated, leptomycin B (LMB) was added to the cell cultures.

Heterokaryon Fusion Assays and Nucleoporin Binding Assays

Fusion assays were performed as described previously (Plafker and Macara, 2000b). Blasticidin-resistant colonies were transduced with G418-sensitive donor and acceptor cells, and plated on poly-L-lysine-coated coverslips. GSN2 cells are stably transfected cells that express a green fluorescent protein (GFP)–streptavidin–nuclear localization signal (NLS) fusion protein (Plafker and Macara, 2000b). Yeast with a HIS3 dominant selectable marker was transformed with the indicated yeast strain and plated as a lawn on medium lacking histidine.

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was added to the media (200 nM). The cells were fixed with 4% (wt/vol) paraformaldehyde (PFA)/PBS, permeabilized with −20°C methanol, and blocked in 3% BSA/PBS.

Nuclear envelope–binding assays were performed essentially as described (Gorlich et al., 1995; Kutay et al., 1997). In brief, factors were assembled in transport buffer (20 mM Hepes-KOH, pH 7.4, 110 mM KOAc, 2 mM MgOAc, 0.5 mM EGTA) before incubation with digitonin-permeabilized BHKB2 cells for 30 min at 24°C. After washing, nuclei were fixed and blocked in 3% BSA/PBS. HA-tagged proteins were detected with 12CA5 monoclonal antibody (1:2,000), Crm1 with a rabbit polyclonal antibody (1:1,000), GST-RanBP3 with a monoclonal anti-GST antibody (1:1,000), and Texas red-conjugated secondary antibodies (1:5,000; Molecular Probes). Nuclei were stained with DAPI. Coverslips were mounted on glass slides with Gel Mount (Biomedia). Cells were visualized by fluorescence microscopy using a Nikon microscope with a 60x water immersion lens. Images were captured with a Hamamatsu charge-coupled device camera and quantified with Openlab software (Improvement).

**Results**

**RanBP3 Increases Crm1 Binding to RanGTP and Export Cargo**

Can Crm1 within a RanBP3–Crm1–RanGTP complex still bind NES cargo? To address this question, we used beads coupled to an MVM-NS2 NES peptide. These beads were exposed to solutions containing combinations of transport factors (Fig. 3 A). Neither GST-RanBP3 nor RanQ69L alone bound significantly to the beads without Crm1 (Fig. 3 A, lane 4), nor did any of these factors bind to control beads associated weakly with Crm1 alone, and RanQ69L substantially increased the binding efficiency, reflecting the increased affinity of Crm1 for NES in the presence of RanGTP (Fig. 3 A, lane 5 vs. 6) (Fornerod et al., 1997a).
When GST-RanBP3 was added to the assay, a small amount bound to Crm1 on the NES beads in the absence of RanQ69L (lane 7). The amount of bound GST-RanBP3 increased when RanQ69L was included (Fig. 3 A, lane 8), most likely reflecting the increased amount of Crm1 bound to the beads. Addition of LMB to the assay abrogated the ability of this complex to bind the beads (Fig. 3 A, lane 9). Therefore, RanBP3 is able to bind in a quaternary complex containing Crm1, RanBP3, NES, and Ran:GTP.

To more quantitatively determine the interaction of Ran:GTP within this complex, we immobilized Crm1-H6 on Ni2+/H1001/NTA beads. The beads were then exposed to a titration of Ran:32P[GTP in the absence or presence of GST-RanBP3 (4:1 molar ratio of RanBP3 to Crm1). The titration was repeated with three levels of NES peptide: a saturating level (40 nM), an intermediate level (100 nM), or no peptide. At the lower levels of NES peptide (100 or 0 nM), RanBP3 significantly increased the amounts of Ran:GTP bound within the complex (Fig. 3 B). These results indicate that, at subsaturating levels of NES, the affinity of the Crm1–RanBP3 complex for Ran:GTP is greater than that of Crm1 alone. At very high NES concentration (40 μM), the binding of Ran-γ32P[GTP to Crm1 was saturable in both the presence and absence of RanBP3. Interestingly, although RanBP3 and Crm1 both contain a Ran-binding domain (RBD), the amount of Ran associated with Crm1 under saturating conditions was identical in either the presence or absence of RanBP3. This experiment indicates that the stoichiometry of Ran:GTP with respect to Crm1 is not increased by binding to RanBP3 and implies that RanBP3 acts by increasing the affinity of the Crm1–RanBP3 complex for Ran:GTP, rather than by independently binding a second Ran:GTP per complex.

It is important to note that the relative concentrations of RanBP3 and Crm1 are critical to the modulation of Ran’s binding to Crm1. At low RanBP3:Crm1 ratios, such as the one used above (4:1), RanBP3 increases the amount of Ran:GTP bound to Crm1. However, when this ratio is increased to 40:1, RanBP3 is unable to increase the amount of Ran:GTP associated with the Crm1 beads (Fig. 3 C). This behavior is predicted from the nature of the Crm1–RanBP3–Ran:GTP complex. A large excess of free RanBP3 is able to compete for available Ran:GTP with the Crm1–RanBP3 complexes present on the beads. The ability to stimulate Ran:GTP binding is dependent on the presence of the RBD of RanBP3, as a mutant that lacks this domain (NF; see below) was unable to mediate the effect (Fig. 3 C).

**Figure 1.** RanBP3 stimulates Crm1-mediated nuclear protein export. (A) Permeabilized HeLa cells were preloaded with bPKI/FITC–STV–NLS export cargo and then exposed to Crm1 (90 nM), Ran (1.0 μM), RanBP3 (1.0 μM), ±LMB (200 nM), as shown. Export activity was monitored as a loss of nuclear fluorescence over 8 min at 30°C. (B) Quantitation of PKI export. Nuclear fluorescence was measured for ~50 randomly chosen cells and expressed as the mean ± 1 SD. Results are representative of two independent experiments. Bar, 10 μm.
The GAP assay is performed in solution, under pseudoequilibrium conditions, whereas bead binding assays involve repeated washing and are not at equilibrium. RanGAP was increased (to 30 nM) to insure complete hydrolysis of free Ran:GTP during the assay. Crm1 alone has a low affinity for Ran:GTP and as expected was unable to protect Ran from the action of RanGAP (Fig. 4 B). However, when RanBP3 or the NES from MVM-NS2 were added to the solution, 63% of the Ran:GTP were protected, respectively. This result indicates that both RanBP3 and NES are able individually to increase the affinity of Crm1 for Ran:GTP. When both RanBP3 and NES were added, the protection was additive, such that 85% of the Ran:GTP were protected, respectively. This result indicates that both RanBP3 and NES are able individually to increase the affinity of Crm1 with Ran:GTP. When both RanBP3 and NES were added, the protection was additive, such that 85% of the GTP remained. Therefore, the formation of a quaternary complex described above (Fig. 3 A) can also be detected in this assay. The effect was not specific to this particular NES, as the increased protection was also observed with the NES from PKI (Fig. 4 B), however to a lesser extent due to the lower affinity of PKI NES versus the MVM NS2 NES for Crm1 (Askjaer et al., 1999).

The fact that NES cargo binds cooperatively with Ran:GTP to Crm1, and that RanBP3 raises the affinity of Crm1 for Ran:GTP (Fig. 3 B), argues that RanBP3 should also increase the affinity of Crm1 for NES cargo. To test this idea, we titrated the RanGAP protection by the MVM-NS2 NES peptide–conjugated beads (5 μl). Bound and unbound fractions were analyzed by anti-His6 (Crm1), anti-Ran (RanQ69L), and anti-GST (GST-RanBP3) immunoblotting. The detected species are indicated at right. (B) Wild-type Ran was loaded with γ[32P]GTP and incubated with immobilized Crm1-H6 (16 pmol) on Ni2+-NTA beads in the absence or presence of GST-RanBP3 (120 nM) or MVM-NS2 NES peptide (0, 100, or 4,000 nM). After washing, bound Ranγ[32P]GTP was counted and graphed as percent maximal binding. (C) Assay was repeated as in B, with either GST-RanBP3 or GST-NF mutant added at indicated molar ratios. Values reported as average of three samples ± SD.
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ter the efficiency of Crm1 association with NES–peptide beads in the absence of Ran (Fig. 3 A). Therefore, within the nucleus, RanBP3 acts to increase the efficiency of Crm1-mediated export by increasing the affinity of the Crm1–RanBP3 complex for Ran:GTP, which cooperatively promotes cargo binding.

The FxFG Domain and RBD of RanBP3 Are Both Required for Efficient Crm1-mediated Export

To better understand the mechanism by which RanBP3 activates Crm1-dependent export, we generated various RanBP3 mutants (Fig. 5 A). The N domain (residues 1–182) contains an NLS; the F domain (residues 183–293) comprises the central region containing two FxFG motifs; the R domain (residues 294–499) comprises the COOH-terminal low-affinity Ran:GTP-binding domain (Radu et al., 1995; Mueller et al., 1998; Welch et al., 1999). Interestingly, we found that RanBP3 possesses two distinct direct binding sites for Crm1 (Fig. 5 B). The major binding site is located in the F domain. A weaker binding site resides in the N domain. No binding was detected using the isolated R domain.

To determine which domains of the RanBP3 protein are responsible for export activity, the export assay was repeated using the recombinant F, R, FR, and NF fragments. Only FR, comprising both the F and R domain, restored full export activity (Fig. 5 C). Remarkably, the F fragment alone had an intermediate activity. However, this activity was suppressed by the presence of the NH2-terminal region, as the NF fragment had no detectable activity. It has been shown that RanBP1 can also stimulate Crm1-mediated export, by dissociating Crm1 from nuclear pores (Kehlenbach et al., 1999). However, it is unlikely that RanBP3 operates via this mechanism, for two reasons. First, the RanBP1 effect on nuclear export is apparent only after preincubation of the cells with RanQ69L (Kehlenbach et al., 1999); second, the R fragment, which contains the RanBP1-like domain, has no activity in this assay (Fig. 5 C).

To determine whether the export activity correlated with the ability of RanBP3 to promote Ran:GTP binding to Crm1, we repeated the RanGAP protection assay with the deletion mutants. As shown in Fig. 5 D, the ability of RanBP3 to promote Ran:GTP binding to Crm1 is dependent on both the F and R domains of RanBP3, matching the requirement for full export activity. Note that the RBD, R, is unable in isolation to promote Ran binding to Crm1, probably because it has a very low intrinsic affinity for Ran:GTP (Mueller et al., 1998).

Together, these data demonstrate that the stimulation of export by RanBP3 requires its major site for Crm1 binding, the F domain, with maximal activity requiring both the F and R domains.

RanBP3 Exports from the Nucleus in a Crm1-dependent Manner

What is the fate of the Crm1–RanBP3–NES-Ran:GTP complex once it has assembled within the nucleus? We considered two alternate hypotheses. First, the quaternary complex could exit the nucleus as a unit. Second, RanBP3 could act as an assembly catalyst and be removed from the complex before export. The first model predicts that
RanBP3 cycles in and out of the nucleus, whereas the second model predicts that it is constitutively nuclear. To distinguish between these models, heterokaryon fusion assays were performed, to test the ability of RanBP3 to shuttle.

For this assay, we used a triple hemagglutinin–tagged RanBP3 (HA3-RanBP3) (Welch et al., 1999). After transfection of BHK21 cells, they were trypsinized and replated with an equal number of GSN2 cells, which express a GFP–streptavidin–NLS fusion protein as a non-shuttling nuclear marker (Black et al., 1999). The two cell types were fused, fixed, and stained. HA3-RanBP3 shuttled from BHK21 nuclei into GSN2 nuclei, as evidenced by the co-staining of the GSN2 nuclei (Fig. 6 A). The GFP–streptavidin–NLS fusion protein remained within the GSN2 acceptor nucleus. We conclude that RanBP3 is a shuttling protein and, in principle, could be exported as a component of a Crm1–RanBP3–NES–Ran:GTP complex.

If this hypothesis were correct, RanBP3 export should be Crm1 dependent. To test this prediction, we used the Crm1-specific inhibitor, LMB. Heterokaryon fusion assays were performed with HA3-RanBP3 and, as a control, with GFP–GFP–RanBP1 (GGRanBP1). RanBP1 is a shuttling protein that contains a LMB-sensitive NES (Richards et al., 1996; Plafker and Macara, 2000a). The additional size imparted by the two GFPs prevents passive diffusion of the protein through the nuclear pores. Therefore, upon the addition of LMB, this construct accumulates irreversibly in the nucleus (Plafker and Macara, 2000a).

HA3-RanBP3 and GGRanBP1 were cotransfected into BHK21 cells that were plated at high density. Adjacent cells were fused, and their cytoplasms were allowed to mix. As expected, GGRanBP1 was cytoplasmic, whereas HA3-RanBP3 was nuclear (Fig. 6 B). When LMB was added after fusion, GGRanBP1 translocated into all of the nuclei of the multikaryon cell. However, when LMB was added before fusion, GGRanBP1 is present only in the donor nuclei, demonstrating that LMB completely prevents the export of a Crm1-dependent cargo. Under these conditions, RanBP3 is unable to shuttle (Fig. 6 B), indicating that export of RanBP3 is LMB sensitive. These data demonstrate that export of RanBP3 is dependent on formation of productive Crm1 export complexes.

The Crm1–RanBP3–NES–Ran:GTP Complex Interacts with NPCs

Since RanBP3 export was Crm1 dependent, we hypothesized that the Crm1–RanBP3–NES–Ran:GTP complex could interact with nucleoporins. To test this hypothesis, we first asked if RanBP3 could compete for Crm1 binding to nucleoporins. Heterokaryon fusion assays were performed with HA3–RanBP3 and, as a control, with GFP–GFP–RanBP1 (GGRanBP1). RanBP1 is a shuttling protein that contains a LMB-sensitive NES (Richards et al., 1996; Plafker and Macara, 2000a). The additional size imparted by the two GFPs prevents passive diffusion of the protein through the nuclear pores. Therefore, upon the addition of LMB, this construct accumulates irreversibly in the nucleus (Plafker and Macara, 2000a).

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The Crm1–RanBP3–NES–Ran:GTP Complex Is Disassembled by RanBP1 and RanGAP

Upon translocation to the cytoplasm, Ran:GTP within the Crm1–RanBP3–NES–Ran:GTP complex must be removed to release NES cargo. What factors mediate this event? It is clear that RanGAP alone is insufficient to mediate disassembly of either the Crm1–Ran:GTP–NES complex (Askjaer et al., 1999) or the Crm1–RanBP3–NES–Ran:GTP complex (Fig. 4). Since RanBP1 has been shown to stimulate export by removing Ran from Crm1–Ran:GTP–NES complexes and subsequent presentation to RanGAP (Askjaer et al., 1999; Kehlenbach et al., 1999), we asked whether it might have a similar role for the Crm1–RanBP3–NES–Ran:GTP complex.

This result was of interest because the F domain contains FxFG motifs that are both representative of nucleoporins and have been shown to directly bind to transport receptors (Radu et al., 1995; Bayliss et al., 2000). Moreover, the F domain is a principal Crm1 binding site on RanBP3.

To determine whether nucleoporins can bind to a Crm1–RanBP3 complex, we used GST-RanBP3 on glutathione–Sepharose beads. In the absence of Crm1, very little Nup214 associated with the beads, suggesting that RanBP3 alone does not bind with high affinity to this nucleoporin. Similarly, when Crm1 was added, it bound RanBP3 without increasing Nup214 binding. However, addition of Crm1 and RanQ69L significantly increased Nup214 binding to the beads. Binding did not occur to beads coupled to GST alone (not shown). These results suggest that RanBP3 and Nup214 can bind simultaneously to Crm1, and this interaction is stabilized by Ran:GTP.

Detergent solubilization destroys the integrity of the NPC, and may obscure low affinity interactions with nucleoporins. We therefore tested the ability of Crm1 to bind intact NPCs in the presence of various transport factors (Fig. 7, C and D) (Fornerod et al., 1997b; Kehlenbach et al., 1999). In the absence of RanBP3, binding of Crm1 to NPCs was readily detectable under all conditions (Kehlenbach et al., 1999) (Fig. 7 C, top). Unexpectedly, RanBP3 inhibited the association of Crm1 with NPCs, and this inhibition was relieved by addition of RanQ69L with or without NES peptide. Conversely, RanBP3 was found to associate with NPCs only in the presence of RanQ69L and NES peptide (Fig. 7 C, bottom). These data suggest that Ran:GTP stimulates binding of the Crm1–RanBP3 complex to NPCs.

To test this hypothesis, we studied the binding of LMB-treated Crm1 to NPCs. In addition to preventing Crm1 from binding to NES-containing proteins, it also prevents high-affinity binding of Crm1 to Ran:GTP (Fornerod et al., 1997a). Since RanBP3 can bind LMB-treated Crm1 (Fig. 2 A), RanBP3 should inhibit binding of LMB-treated Crm1 to NPCs even in the presence of RanQ69L or RanQ69L and NES peptide. This effect was observed (Fig. 7 C, bottom, and D), demonstrating that high-affinity Ran binding to Crm1 stimulates the Crm1–RanBP3 complex to bind NPCs.

Figure 6. RanBP3 exports from the nucleus in a Crm1-dependent manner. (A) Triple HA-tagged RanBP3 (HA₃–RanBP3) was transiently expressed in BHK21 cells, which were trypsinized and replated with an equal number of GSN2 cells and then fused by addition of polyethylene glycol. Cells were fixed and stained with anti-HA Texas red–conjugated secondary antibodies. Nuclei were identified by DAPI staining. (B) BHK21 cells were cotransfected with vectors encoding HA₃–RanBP3 and GGGRanBP1 before cell–cell fusion. Cells were incubated with LMB (200 nM) after fusion, or before and after fusion, or with no LMB. After 1 h, cells were prepared as described above. Bars, 5 μm.
To test this hypothesis, RanGAP was added to preassembled export complexes with or without RanBP1. RanBP1 and RanGAP, but not RanGAP alone, were able to hydrolyze Ran:GTP bound within both the Crm1–Ran: GTP–NES complex as well as the Crm1–RanBP3–NES– Ran:GTP quaternary complex (Fig. 8 A).

What is the mechanism by which RanBP1 disassembles the quaternary complex? The RBDs of RanBP1 and RanBP3 are related structurally, and it is likely that RanBP1 would compete with the RBD of RanBP3 within the complex to gain access to Ran:GTP. To test this idea, glutathione–Sepharose beads coated with GST–RanBP3 were incubated with excess Crm1, RanQ69L, and NES peptide (Fig. 8 B). Under these conditions, no detectable RanQ69L is able to bind RanBP3 in the absence of Crm1 (data not shown). After complex assembly, the beads were washed then resuspended in either buffer alone or buffer containing RanBP1 (1 μM), and the bound and unbound fractions were analyzed at 1 and 10 min. At 1 min, a slightly greater amount of Crm1 was released from the beads in the presence of RanBP1 (ratio bound/total = 0.59 vs. 0.52). At 10 min the difference was greater, with the ratio decreasing from 0.52 to 0.39. The effect of RanBP1 on the release of Ran from this complex was more pronounced. At 10 min, ~90% of the RanQ69L was released with RanBP1, whereas in contrast only ~50% was released without RanBP1. These results indicate that RanBP1 can remove Ran:GTP from the Crm1–RanBP3–NES–Ran:GTP quaternary complex.

**Discussion**

In this study we have shown that RanBP3 is a cofactor for Crm1-mediated nuclear protein export. RanBP3 binds directly and specifically to Crm1. RanBP3 binding increases the affinity of the Crm1–RanBP3 complex for Ran:GTP.
Based on these data, we propose a working model for the facilitation of nuclear protein export by RanBP3 (Fig. 9). In this model, RanBP3 interacts with nuclear Crm1 and Ran-GTP to facilitate cargo loading. The RBD of RanBP3 stabilizes the interaction of Crm1 with Ran-GTP, which cooperatively increases the affinity of Crm1 for NES-containing cargo and for the NPC. The association of Ran and NES with Crm1 induces a conformational switch, altering the binding mode to RanBP3. The switch reduces interaction of Crm1 with the F domain, but binding through the N domain of RanBP3 prevents complex disassembly. Additionally, Ran-GTP mediates an interaction between Crm1 and the R domain of RanBP3. The conformational switch in Crm1 also exposes a nucleoporin-binding site. We speculate that the F domain may obscure this site in the absence of Ran-GTP and cargo, thereby reducing binding to the NPC. We suggest that the quaternary export complex translocates through the pore by a facilitated diffusive process that requires the low affinity transient association of Crm1 with nucleoporin FxFG repeats (Ribbeck and Gorlich, 2001). On the cytoplasmic side of the NPC, RanBP1 liberates Ran-GTP and presents Ran-GTP to RanGAP. This terminal step may be promoted by another cofactor, Nxt1, which appears to facilitate the release of Crm1 and cargo from the NPC (Black et al., 2001). RanBP3 and Crm1 can then return to the nucleus. It will be of interest to determine whether in fact RanBP3 either facilitates the reimport of Crm1 via the importin α/β pathway, or whether Crm1 can facilitate the reimport of RanBP3. Either or both mechanisms seem plausible, unless there is an efficient mechanism in the cytosol to ensure dissociation of the RanBP3–Crm1 complex.

The model predicts that RanBP3 reduces the association of Crm1 with the NPC. Crm1 alone does show detectable binding to NPCs, which is inhibited by RanBP3 (Fig. 7 C). However, binding of Crm1 to solubilized nucleoporins in vitro (Fig. 7 A) was not detectable either in the presence or absence of RanBP3. This difference may be a result of the dilution of nucleoporins during solubilization, to interference by the detergent, to the existence of Crm1 binding sites not detected by the RL-1 antibody, or to loss of higher order structures. Dilution or detergent interference are perhaps unlikely because we have been unable to detect significant binding of Crm1 to recombinant nucleoporin p62 in the absence of this detergent (and of Ran-GTP) in bead binding assays. At present, therefore, we consider it likely that NPCs possess Crm1 binding sites in addition to Nup214 and p62 that are not detectable by the RL-1 antibody, or that there are sites destroyed by solubilization.

The RBD of RanBP3 is related to those of RanBP1 and RanBP2–Nup358 (Mueller et al., 1998), and the ability to potentiate Crm1-mediated RanGAP protection was, therefore, unexpected. In fact, these two RBDs have similar activities in isolation (Beddow et al., 1995; Bischoff et al., 1995; and Fig. 4 A), but have opposing activities in the context of karyopherin family members. Although RanBP1 stimulates the RanGAP-mediated hydrolysis of Ran-GTP in complex with Crm1, RanBP3 has the reverse effect and substantially inhibits RanGAP activity towards a Crm1–RanBP3–Ran-GTP ternary complex. This effect depends, however, on the relative concentrations of the RanBP3 and Crm1. When present at a large molar excess,
free RanBP3 will compete with the Crm1–RanBP3 complex for Ran:GTP and will stimulate RanGAP activity. We have observed this behavior in RanGAP assays (data not shown) and binding assays, and it may explain the finding that overexpression of the RanBP3 related protein, Yrb2p, is toxic to yeast (Taura et al., 1997).

Our data are also consistent with other studies of YRB2 function. Although XPO1 is an essential gene in yeast, strains containing a null allele of YRB2 are viable, suggesting that Yrb2p, like RanBP3, facilitates, but is not necessary for Xpo1p–Crm1p-dependent nuclear protein export (Taura et al., 1998; Noguchi et al., 1999). The forced overexpression of Gsp1p (yeast Ran) or Xpo1p suppresses the cold sensitivity of the expression of Gsp1p (yeast Ran) or Xpo1p (Taura et al., 1998; Noguchi et al., 1999). The forced overexpression of the RanBP3 related protein, Yrb2p, is toxic to yeast (Taura et al., 1997).

Finally, the recent discovery of new cofactors for Crm1-mediated export—Nxt1, elf5A, and RanBP3—exemplify the complexity of nuclear transport mechanisms and suggest that the search for cofactors involved in other nucleocytoplasmic transport systems may be a fruitful avenue of research.

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Figure 9. A model for RanBP3-assisted nuclear protein export. RanBP3 interacts with nuclear Crm1. The RBD of RanBP3 stabilizes the interaction of Crm1–RanBP3 and Ran:GTP, increasing the affinity of the complex for NES-containing cargo. Ran:GTP binding to Crm1–RanBP3 also releases inhibition to pore binding. The Crm1–RanBP3–NES–Ran:GTP cargo complex translocates through the pore. On the cytoplasmic side of the pore, RanBP1 removes Ran:GTP allowing presentation to RanGAP, terminating export.
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