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 possesses 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 quarternary 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)[1], 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 (Matunis 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; Ossarehnazari 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

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 quarternary 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 pKH3 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 Dirk Görlich (University of Heidelberg, Heidelberg, Germany), and p62 cDNA from John Hanover (National Institutes of Health, Bethesda, MD). All constructs were checked by automated sequencing. Peptides used in binding and GAP assays: MVM NS2 NES peptide, CVDEMT-KKFGTLTIHDTEK (Askjaer et al., 1999); PKI NES peptide, GSNELA-LKLAGLDINKTGGC (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* XL1-Blue as described previously (Lounsbury 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 CaCl$_2$) 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$_6$ 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 Centricon 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$_6$ 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. Gerace, The Scripps Research Institute, La Jolla, CA; 1:10,000), 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 Ni^{2+}-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-H$_6$ 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 H$_2$SO$_4$ and read at 410 nM on a EL$_X$-800 plate reader (Bio-Tek Instruments). Binding data were fit by an iterative procedure to an equation describing a single binding site: fractional saturation, $f = x/(K_{app} + x)$ (where x = Crm1 for immunoblotting and GST-RanBP3 for ELISA). Analysis was performed using Kaleidagraph 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 MVM 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 *HIS3* gene driven by the Gal1 promoter allowing for Gal4 fusion protein–dependent histidine prototrophy. The yeast were mated with W303α strains harboring fusion proteins of the VP16 activation domain and Ran or RanBP3 or vector alone, and then replica plated onto L$^-$H$^-$W$^-$ drop-out medium to select for bait–prey interactions (Plafker and Macara, 2000b).

Heterokaryon Fusion Assays and Nucleoporin Binding Assays

Fusion assays were performed as described previously (Plafker and Macara, 2000b). Baby hamster kidney (BHK21) cells were transfected by the CaPO$_4$ method (Sambrook et al., 1989). After 24 h, these donor cells were trypsinized, mixed with GSN2 acceptor cells, and replated onto 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 (Black et al., 1999). At 48 h, the cells were fused by the addition of 40% PEG 3500 in HBSS for 2 min at 37°C. 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 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 BHK21 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 60× water immersion lens. Images were captured with a Hamamatsu charge-coupled device camera and quantified with Openlab software (Improvision).

RanGAP Assays

RanGAP protection assays were performed as described (Askjaer et al., 1999). In brief, Ran was loaded with $\gamma[^{32}P]GTP$ (Lounsbury and Macara, 1997) and added to Crm1 (330 nM), ±NES peptide (0–20 μM), and ±RanBP3 (1 μM) in 40 μl reaction buffer (36 mM Tris-HCl, pH 8.0, 75 mM NaCl, 6 mM $MgCl_2$, 0.8 mM dithiothreitol, 0.5 mM GTP, 0.1 mg BSA/ml, 1 mM $NaPO_4$, and 1% glycerol) for 20 min on ice. Diluted GST-RanGAP was added to the complexes at 34°C for 4 min. The reaction mixes were passed through nitrocellulose filters and washed. Ran:$\gamma[^{32}P]GTP$ was quantitated by scintillation counting. Values are expressed as the percentage of counts bound after incubation of Ran without RanGAP.

In Vitro Protein Export Assay

Nuclear export assays were performed as described previously with RanBP3 construct supplemented in the 8-min export phase (Holaska and Paschal, 1998). Recombinant proteins used were produced as described above. Quantitative data represent a minimum of 40–50 cells per reaction.

Results

RanBP3 Stimulates Crm1-mediated Nuclear Protein Export

Genetic and biochemical evidence has linked the yeast orthologue of RanBP3, Yrb2p, to Xpo1p-dependent nuclear protein export, but the function of Yrb2p in export remains unknown (Taura et al., 1998; Noguchi et al., 1999). To test whether RanBP3 is involved in Crm1-mediated nuclear export, we used an in vitro nuclear export assay that uses the NES of PKI (Holaska and Paschal, 1998). This two-step assay involves an import and an export phase. In the import phase, a fluorescein isothiocyanate–conjugated streptavidin–NLS fusion protein (FITC–STV–NLS) is imported into nuclei of digitonin-permeabilized HeLa cells with lysate. In the export phase, biotinylated PKI (bPKI) is added, which is small enough to diffuse into the nucleus. There it can bind the fluorescent streptavidin and, in the presence of soluble transport factors, mediate export of bPKI/FITC–STV–NLS into the cytoplasm. Residual nuclear fluorescence is quantitated, and export activity is measured as the loss of fluorescence compared with controls that lack the necessary transport factors.

To seek an effect of RanBP3 on nuclear protein export, it was necessary to find levels of Crm1 and Ran that were limiting with respect to export activity. A limiting concentration of Crm1 (90 nM) alone or Crm1 plus Ran (1.0 μM) did not significantly increase the amount of export over buffer alone (Fig. 1, A and B). However, when this amount of Crm1 and Ran was supplemented with GST-

RanBP3 (1.0 μM), a significant loss of nuclear fluorescence was measured. This activity was fully inhibited by the addition of LMB, which reacts covalently with Crm1 and inhibits its interaction with NES cargo (Kudo et al., 1999). RanBP3 lacking the GST tag also activated export (data not shown). Therefore, RanBP3 can stimulate Crm1-dependent export activity in a permeabilized cell nuclear export assay.

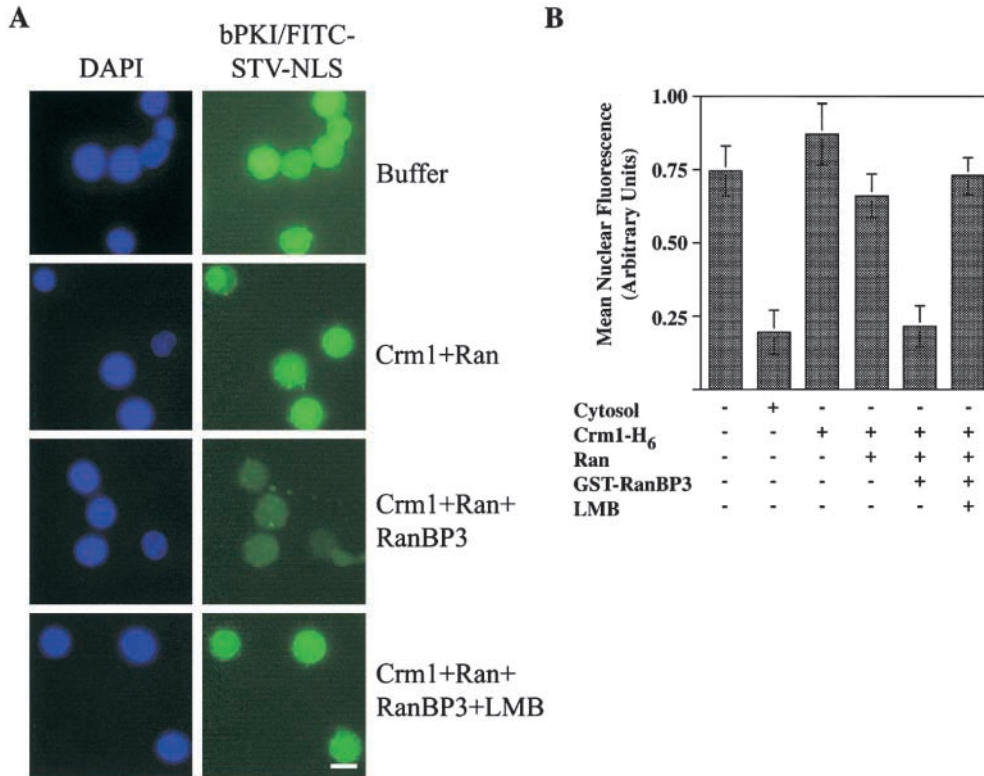### RanBP3 Binds Directly and Specifically to Crm1

Next, we asked whether a direct association could be observed between RanBP3 and Crm1. As shown in Fig. 2 A, His-tagged Crm1 (Crm1-H_6) associated with GST-RanBP3 directly (lane 8). RanQ69L, a GTPase-deficient Ran mutant, was efficiently incorporated into the Crm1–RanBP3 complex without reducing the levels of bound Crm1 (lanes 8 and 9). These data suggest that Crm1, RanBP3, and Ran can form a stable ternary complex.

Crm1 has been shown to form ternary complexes with Ran:GTP and NES-bearing proteins. We predicted that RanBP3 was not acting as a classic NES, because it could enhance Crm1-mediated export in vitro (Fig. 1) and its binding to Crm1 was not enhanced by Ran. Nevertheless, to determine whether binding of RanBP3 to Crm1 was competitive with NES binding, we analyzed the Crm1–RanBP3 interaction in the presence of a large molar excess of a NES peptide. Neither NES peptide alone nor NES peptide plus RanQ69L prevented Crm1 from binding GST-RanBP3 (Fig. 2 A, lanes 10 and 11). Moreover, LMB did not inhibit binding of Crm1 or RanQ69L to GST-RanBP3 (lane 12), although it completely inhibited Crm1 binding to NES (Fig. 3 A). Together, these data demonstrate that the interaction between Crm1 and RanBP3 is distinct from that between Crm1 and NES.

To assess the selectivity of this interaction, the binding of RanBP3 to other karyopherin-β family members was assayed in a yeast dihybrid assay. Only strains harboring Crm1 and RanBP3 were able to support growth in the absence of histidine (Fig. 2 B). Importin-11, CAS, and importin-β were functional in this assay as they were able to interact with VP16-Ran. The poor ability of Crm1 to interact with Ran in this assay has been reported by others (Stade et al., 1997). These data suggest that RanBP3 may be a specific partner of Crm1.

RanBP3 Increases Crm1 Binding to Ran:GTP and Export Cargo

Can Crm1 within a RanBP3–Crm1–Ran:GTP 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 generated from an unrelated peptide encoding an NH_2-terminal sequence from the small GTPase TC10 (Fig. 3 A, lanes 1–3). As expected from previous work, the NES 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 Ran:GTP (Fig. 3 A, lane 5 vs. 6) (Fornerod et al., 1997a).

A

DAPI | bPKI/FITC-STV-NLS

Buffer

Crm1+Ran

Crm1+Ran+RanBP3

Crm1+Ran+RanBP3+LMB

B

Cytosol	-	+	-	-	-	-
Crm1-H$_6$	-	-	+	+	+	+
Ran	-	-	-	+	+	+
GST-RanBP3	-	-	-	-	+	+
LMB	-	-	-	-	-	+

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 in each sample and expressed as the mean ± 1 SD. Results are representative of two independent experiments. Bar, 10 μm.

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 quarternary complex containing Crm1, RanBP3, NES, and Ran:GTP.

To more quantitatively determine the interaction of Ran:GTP within this complex, we immobilized Crm1-H$_6$ on Ni^{2+}-NTA beads. The beads were then exposed to a titration of Ran:γ[^{32}P]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 μM), 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:γ[^{32}P]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).

RanBP3 Increases the Affinity of Crm1 for NESs

To determine the effect of RanBP3 on the binding of cargo to Crm1, we performed RanGAP protection assays. However, in light of the previously reported ability of Yrb2p to weakly coactivate RanGAP (Noguchi et al., 1997), we first tested whether RanBP3 had a similar biochemical activity. Using a low concentration of RanGAP (150 pM) such that only ∼25% of the total Ran-bound γ[^{32}P]GTP was hydrolyzed within the time frame of the assay, we observed that RanBP3 has a weak ability to coactivate RanGAP in the absence of other factors (Fig. 4 A). However, when Crm1 and NES were added to the assay, Ran was protected from the action of RanGAP (data not shown).

The ability of the quaternary complex to protect Ran from the action of RanGAP allowed us to assess quantitatively the interaction of Ran and NES with Crm1. The advantage of the GAP protection assay over the direct bind-
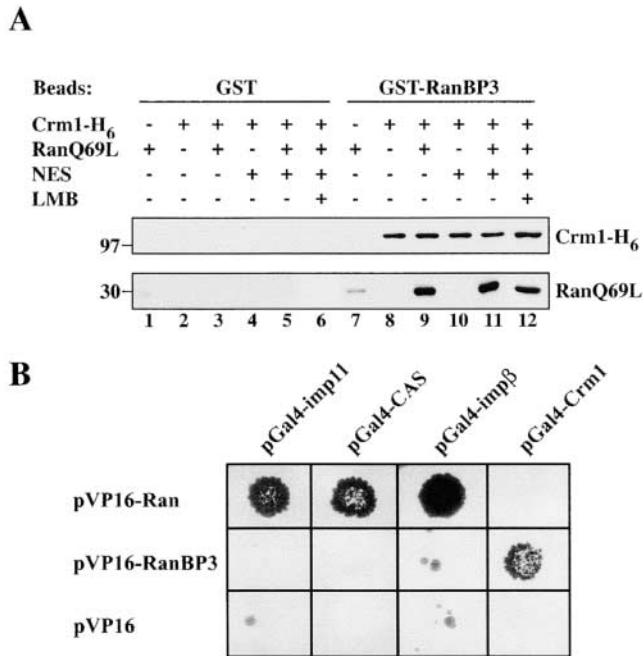
Figure 2. Crm1 binds directly and specifically to RanBP3. (A) Crm1 binds directly to RanBP3 in the presence of RanQ69L and NES peptide. Recombinant Crm1-H$_6$ (200 nM) was incubated with GST-tagged RanBP3 attached to glutathione–Sepharose beads in the presence of RanQ69L (2 μM), MVM NES peptide (2 μM), or LMB (2 μM), as shown, and bound protein was analyzed by immunoblotting. (B) Yeast dihybrid conjugation assays. *S. cerevisiae* HF7c (MAT-a) that express bait (GAL4DBD) fusions of importin-11, Cas, importinβ, and Crm1 were mated to *S. cerevisiae* W303 (MAT-α) that express the VP16 activation domain alone or as prey fusions with Ran or RanBP3. Growth of diploids is shown after replica plating to L⁻H⁻W⁻ plates, which support growth only of those yeast in which the bait fusion interacts with the prey fusion protein.

ing measurements shown in Fig. 3 is that 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, ∼25 and ∼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 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).
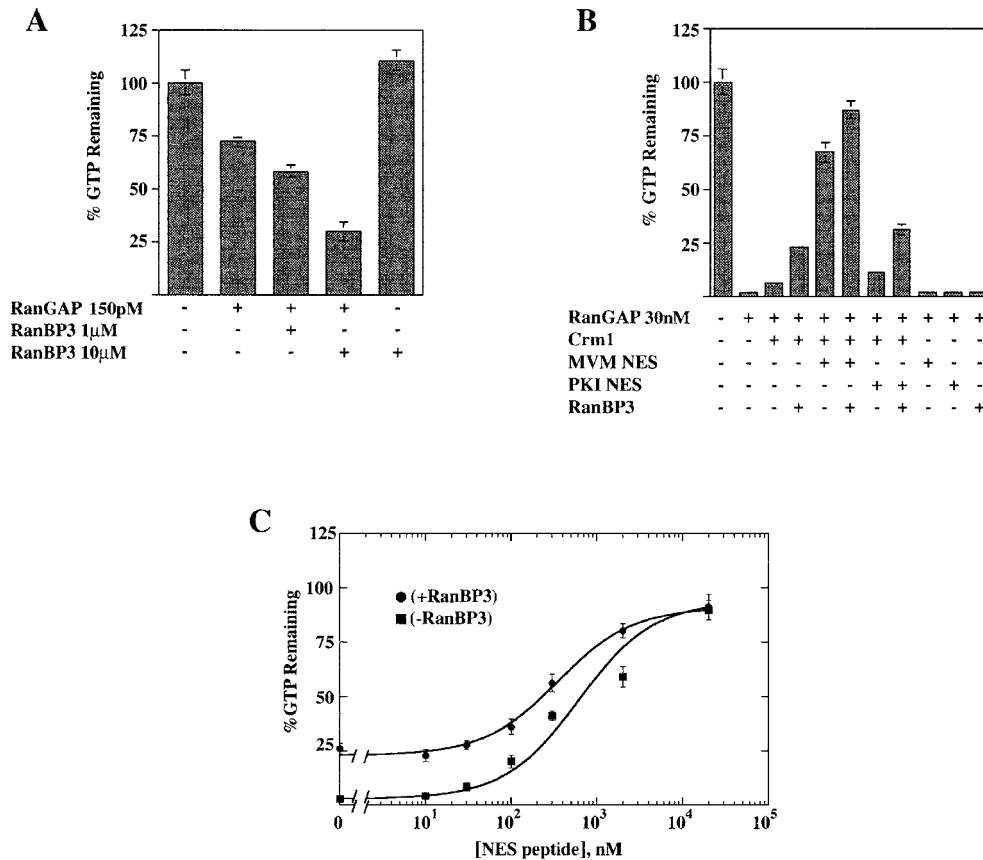
The fact that NES cargo binds cooperatively with Ran:GTP to Crm1, and that RanBP3 raises the affinity of Crm1 for Ran:GTP (Fig. 3 B), argues that RanBP3 should also
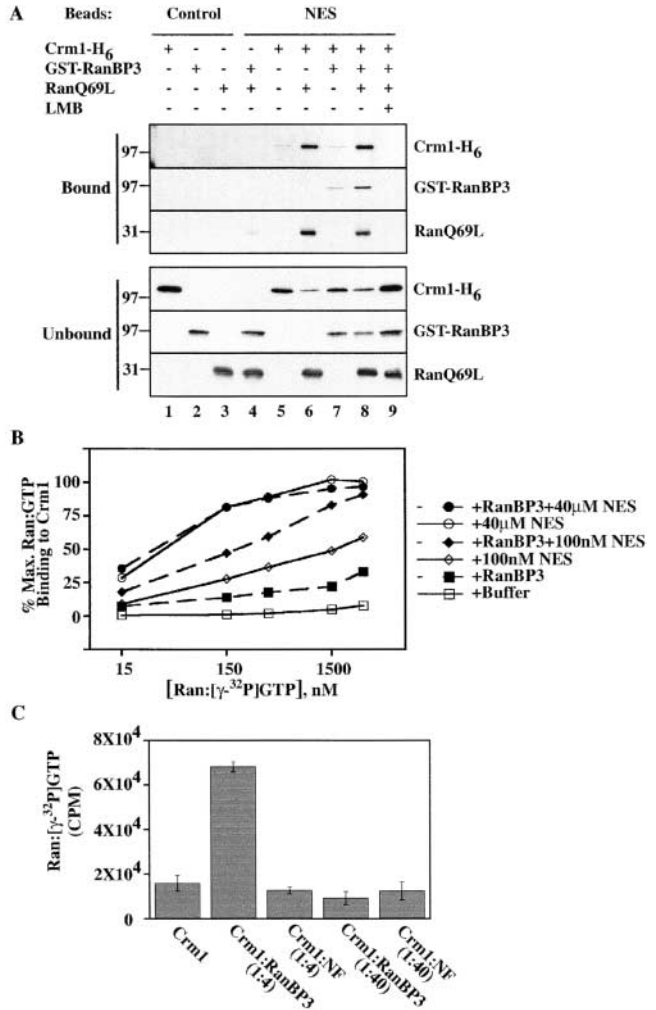
Figure 3. RanBP3 increases the affinity of Crm1 for Ran:GTP. (A) Various combinations of Crm1-H$_6$ (330 nM), GST-RanBP3 (250 nM), and RanQ69L (2.0 μM) were incubated with MVM NS2 NES peptide–conjugated beads (5 μl). Bound and unbound fractions were analyzed by anti-His$_6$ (Crm1), anti-Ran (RanQ69L), and anti-GST (GST-RanBP3) immunoblotting. The detected species are indicated at right. (B) Wild-type Ran was loaded with γ[^{32}P]GTP and incubated with immobilized Crm1-H$_6$ (16 pmol) on Ni^{2+}-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[^{32}P]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.

increase the affinity of Crm1 for NES cargo. To test this idea, we titrated the RanGAP protection by the MVM-NS2 NES in the absence and presence of RanBP3. Increasing the concentration of NES increased the protection from RanGAP, in a saturable manner (Fig. 4 C), and RanBP3 was able to increase the affinity of Crm1 for the MVM-NS2 NES by 1.6-fold (from $K_{app} \approx 540$–330 nM).

These data indicate that RanBP3 and NES are both able to facilitate Ran:GTP binding to Crm1 complexes, but by independent mechanisms. RanBP3 also increases the affinity of Crm1 for NES cargo, but this property seems to be dependent on Ran binding because RanBP3 did not al-

A

RanGAP 150pM	-	+	+	+	-
RanBP3 1μM	-	-	+	-	-
RanBP3 10μM	-	-	-	+	+

B

RanGAP 30nM	-	+	+	+	+	+	+	+	+	+	+
Crm1	-	-	+	+	+	+	+	+	-	-	-
MVM NES	-	-	-	-	+	+	-	-	+	-	-
PKI NES	-	-	-	-	-	-	+	+	-	+	-
RanBP3	-	-	-	+	-	+	-	+	-	-	+

C

- ● (+RanBP3)
- ■ (−RanBP3)

[NES peptide], nM

Figure 4. RanBP3 increases the affinity of Crm1 for Ran:GTP and NES. (A) RanBP3 is a weak coactivator of RanGAP. GST-Ran-GAP (0.15 nM) was added to preassembled complexes of Ranγ[^{32}P]GTP (40 nM) +RanBP3 (1 or 10 μM) for 4 min at 30°C. After filter binding to nitrocellulose, protein-associated Ranγ[^{32}P]GTP was detected by scintillation counting. (B) RanBP3 increases Crm1-mediated protection of Ran:GTP from RanGAP. GST-RanGAP (30 nM) was added to pre-assembled complexes of Ranγ[^{32}P]GTP (40 nM), cleaved RanBP3 (1 μM), Crm1-H$_6$ (330 nM), and NES peptide (200 nM). (C) RanBP3 increases the Crm1 affinity for NES. Titration of Crm1 and Ranγ[^{32}P]GTP with an increasing concentration of NES peptide (0–20 μM) was performed in the presence (●) or absence (■) of RanBP3 (1 μM).

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 NH$_2$-terminal region, as the NF fragment had no detectable activity. It has been shown that RanBP1 can also stimulate Crm1-medi-

ated 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 quarternary 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
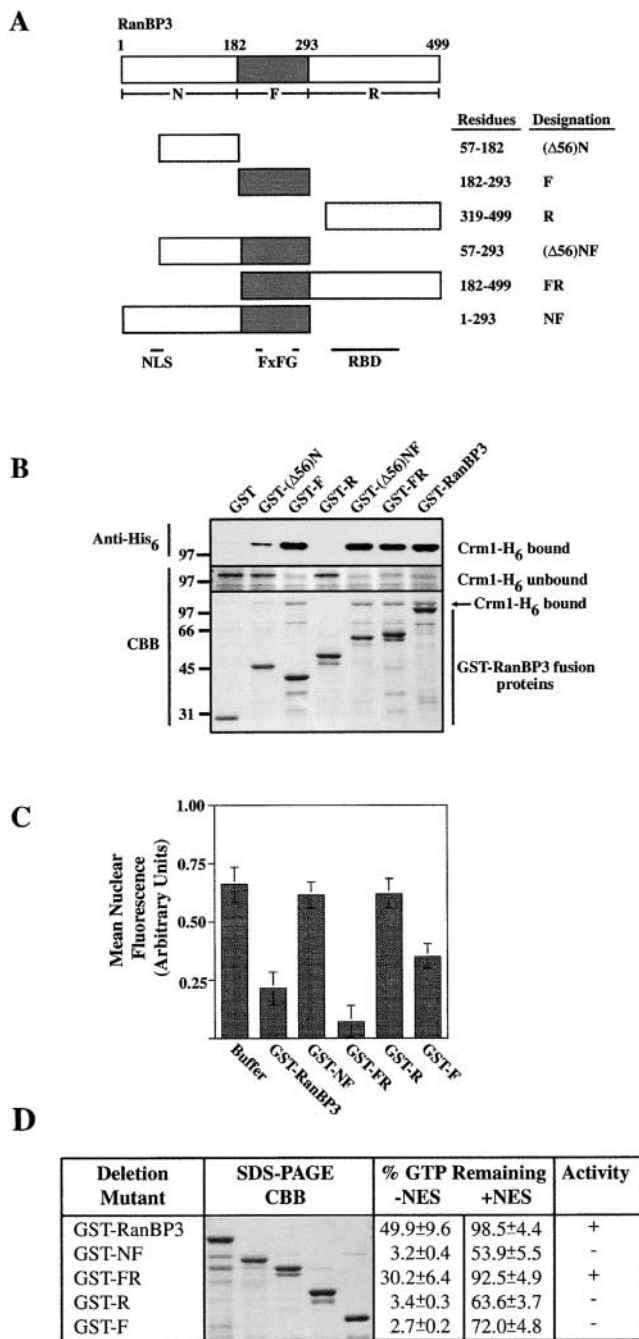
Figure 5. The FxFG-containing domain and RBD of RanBP3 are required for full export activity. (A) Diagram of deletion mutants, showing domain structure of RanBP3. Mutants are illustrated without the 27-kD amino terminal GST tag. Locations of the NLS, two FxFG repeats, and the RBD are indicated. (B) Crm1-H$_6$ was exposed to glutathione–Sepharose beads coated with various GST-tagged RanBP3 fragments. Unbound protein was identified by Coomassie brilliant blue (CBB) staining. Bound protein was identified by both Coomassie staining and immunoblotting with an anti-His$_6$ antibody. (C) Deletion mapping of Crm1-dependent export activity. The PKI export assay was performed as described in the legend to Fig. 1, using 100 μg/ml of each GST-RanBP3 fragment. Nuclear fluorescence was quantitated in ~50 randomly chosen cells for each experimental condition and is presented as the mean ± SD. Export activity is represented as loss of fluorescence. (D) The RanGAP protection assay was repeated as described in the legend to Fig. 4 with equimolar levels of GST-RanBP3 deletion mutants.

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 (HA$_3$-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. HA$_3$-RanBP3 shuttled from BHK21 nuclei into GSN2 nuclei, as evidenced by the costaining 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 HA$_3$-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).

HA$_3$-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 HA$_3$-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. Cells were permeabilized with digitonin, washed to remove soluble components, and then solubilized with 1% NP-40 (Kehlenbach et al., 1999). The soluble extract, enriched in nucleoporins, was incubated with Crm1 and/or RanBP3, RanQ69L, and NES peptide. Crm1 was precipitated with anti-Crm1 antibodies, and associated proteins were detected by immunoblotting. When supplemented with RanQ69L or RanQ69L and NES peptide, Crm1 bound both Nup214 and p62 (Fig. 7 A), as reported previously (Kehlenbach et al., 1999). When RanBP3 was added in large excess, it did not reduce nucleoporin binding, suggesting that RanBP3 does not compete with Nup214 or p62 for Crm1 in the presence of Ran:GTP.
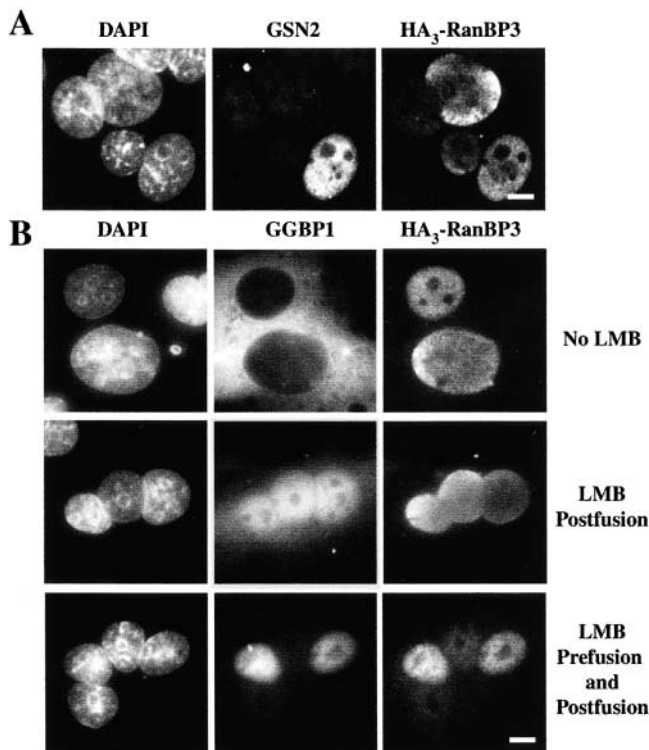
Figure 6. RanBP3 exports from the nucleus in a Crm1-dependent manner. (A) Triple HA-tagged RanBP3 (HA₃-RanBP3) was transiently expressed in BHK21 cells, which were trypsinized and replated with an equal number of GSN2 cells and then fused by addition of polyethyleneglycol. Cells were fixed and stained 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 GGRanBP1 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.

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 (Kehlen-

bach 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 or 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.

How might Ran:GTP modulate the binding of the Crm1–RanBP3 complex to the NPC? One interesting possibility is that Ran might alter the conformation of Crm1 such that it interacts with RanBP3 in a different manner, exposing a surface on Crm1 involved in NPC binding. To study the Crm1–RanBP3 interaction further, we first determined its affinity by ELISA and quantitative immunoblotting. Each of these methods produced nearly identical values (data not shown). By ELISA, the apparent dissociation constant of the RanBP3-Crm1 complex was 64 ± 14 nM; this affinity was reduced only slightly by Ran:GTP and NES (106 ± 23 nM) (data not shown). A control peptide that does not bind Crm1 (Fig. 3 A) does not mediate this reduction. The cause of this change in affinity became evident when we tested the effect of Ran:GTP on binding of Crm1 to isolated domains of RanBP3. Ran:GTP plus NES substantially reduced binding of the F domain of RanBP3 to Crm1, although it increased binding of the R domain (Fig. 7 E). Together, these data suggest that, in the absence of Ran:GTP and NES, RanBP3 binds to Crm1 principally through the N and F domains, whereas in the presence of Ran:GTP and NES, the interaction occurs primarily through the N and R domains. Therefore, although the affinity of full length RanBP3 for Crm1 does not change substantially, there is a significant change in the mode of binding. This change in binding mode could explain how Ran alters the ability of the RanBP3-Crm1 complex to interact with NPCs (see Discussion).

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.
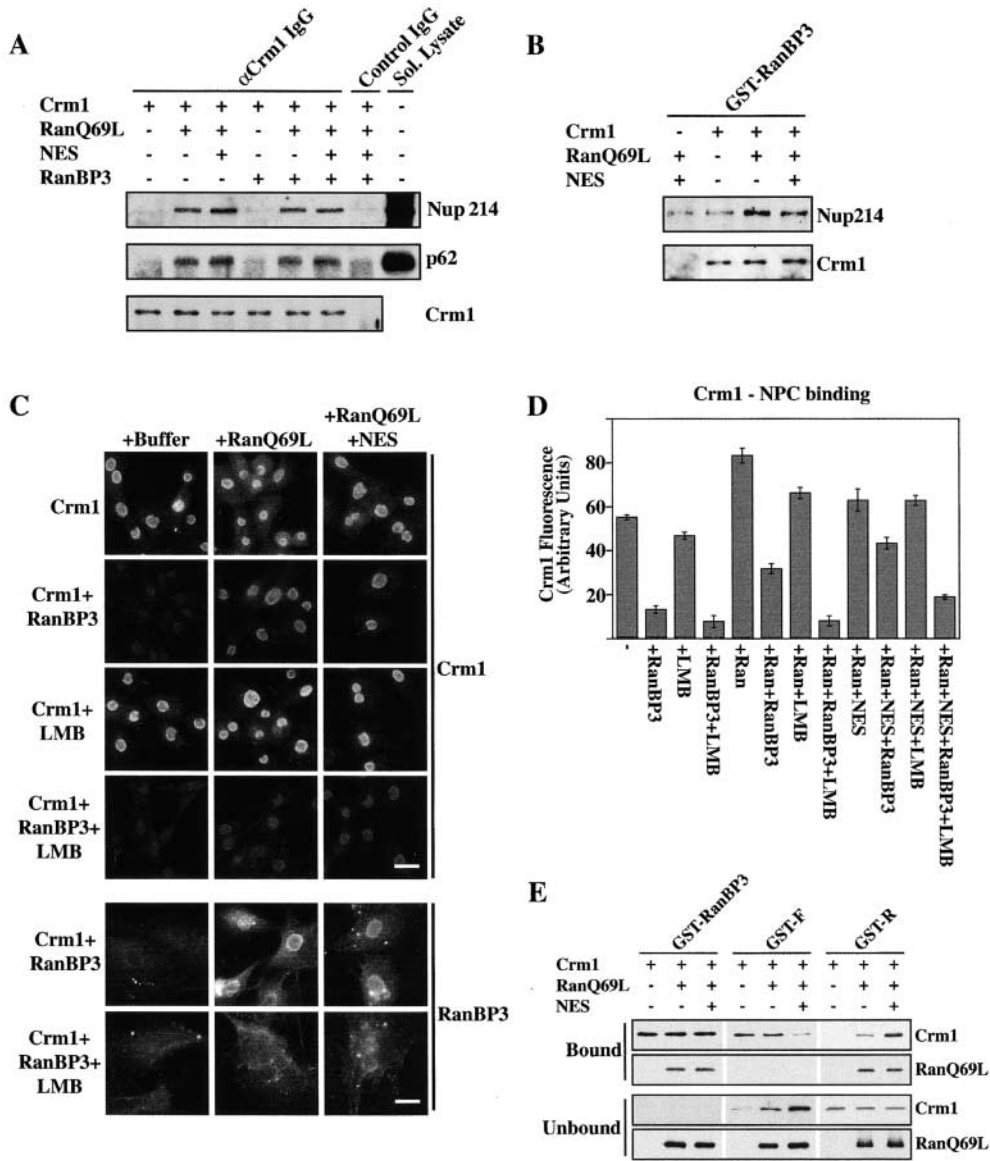
Figure 7. The Crm1–RanBP3–NES–Ran:GTP complex interacts with the NPC. (A) Crm1 immunoprecipitations were performed in the presence or absence of excess RanBP3. 4×10^8 digitonin-permeabilized HeLa cells were solubilized, after which the indicated transport factors were added, incubated for 1 h at 4°C, and precipitated with anti-Crm antibody and protein A–Sepharose for 1 h. After washing 4× in solubilization buffer, bead associated proteins were immunoblotted with RL-1 antibody (Nup214) or anti-p62 antibody. (B) The assay in C was repeated using GST-RanBP3 to precipitate Crm1. (C) NPC binding assays. Crm1-H_6 (40 nM) and/or GST-RanBP3 (400 nM) were overlayed onto digitonin-permeabilized BHK21 cells with either RanQ69L (2 μM), MVM-NS2 NES peptide (4 μM), or LMB (2 μM) at 24°C for 30 min. Crm1 and RanBP3 were identified by indirect immunofluorescence. Bar, 20 μm. (D) Quantitation of Crm1 nuclear fluorescence shown in C. Data represent 50–60 nuclei per sample ± 1 SEM. (E) Crm1 binding to RanBP3 in the presence of Ran:GTP. Crm1 (500 nM) was incubated with GST-RanBP3, GST-F, or GST-R on glutathione–Sepharose ± RanQ69L (2 μM) and MVM NS2 NES peptide (4 μM), as shown, and bound or unbound proteins were immunoblotted.

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

A

B
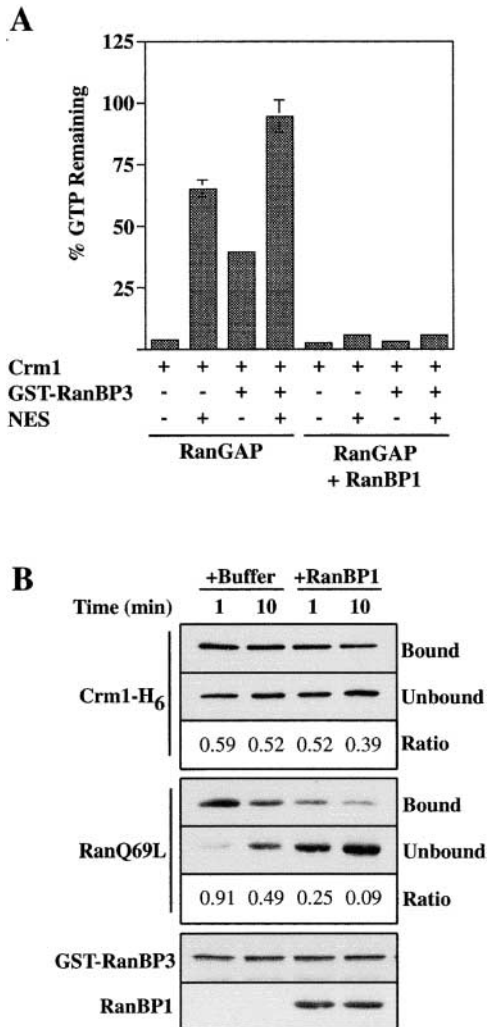
Figure 8. The Crm1–RanBP3–NES–Ran:GTP complex is disassembled by RanBP1 and RanGAP. (A) GST-RanGAP (30 nM) or GST-RanGAP plus RanBP1 (1.0 µM) were added to complexes of Ranγ[^{32}P]GTP (40 nM), GST-RanBP3 (1.0 µM), Crm1-H$_6$ (330 nM), and MVM-NS2 NES peptide (200 nM). After 4 min at 34°C, samples were bound to nitrocellulose filters and washed. Filter-bound Ranγ[^{32}P]GTP was detected by scintillation counting. (B) Glutathione–Sepharose beads loaded with GST-RanBP3, Crm1-H$_6$, RanQ69L, and MVM-NS2 NES peptide, were aliquoted and resuspended in either buffer or buffer containing RanBP1 (1.0 µM). Samples of bound and unbound proteins were analyzed by immunoblotting.

and NES. RanBP3 possesses three domains: an NH$_2$-terminal region, N, that contains a NLS (Welch et al., 1999); an F domain that contains two FxFG nucleoporin-like motifs; and an R domain that is similar to the RBD of RanBP1. We found that the N and F domains each possess direct binding sites for Crm1 but that the F and R domains are both required for full export activity. Additionally, we found that RanBP3 is a shuttling protein and that its export is inhibited by the Crm1-specific inhibitor, LMB. The Crm1–RanBP3 complex exhibits Ran:GTP-dependent interactions with nucleoporins and with the NPC. Finally, we have shown that the Crm1–RanBP3–NES–Ran:GTP quaternary complex can be disassembled by the action of RanBP1 and RanGAP.

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,
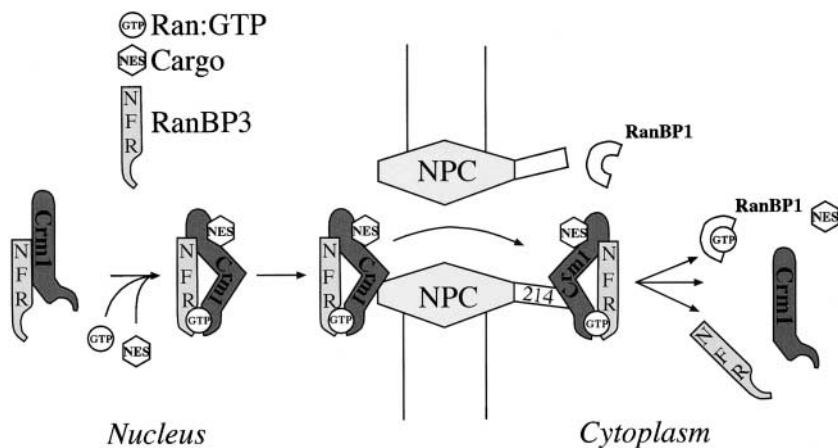
Figure 9. A model for RanBP3-assisted nuclear protein export. RanBP3 interacts with nuclear Crm1. The RBD of RanBP3 stabilizes the interaction of Crm1–RanBP3 and Ran: GTP, increasing the affinity of the complex for NES-containing cargo. Ran:GTP binding to Crm1–RanBP3 also releases inhibition to pore 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.

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 *yrb2Δ* null mutant (Noguchi et al., 1999), suggesting that an increase in the nuclear concentration of either of these proteins can drive the formation of a competent export complex even in the absence of Yrb2p.

Finally, the recent discovery of new cofactors for Crm1-mediated export—Nxt1, eIF5A, and RanBP3—exemplify the complexity of nuclear transport mechanisms and suggest that the search for cofactors involved in other nucleo-cytoplasmic transport systems may be a fruitful avenue of research.

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