RanBP3 influences interactions between CRM1 and its nuclear protein export substrates

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We investigated the role of RanBP3, a nuclear member of the Ran-binding protein 1 family, in CRM1-mediated protein export in higher eukaryotes. RanBP3 interacts directly with CRM1 and also forms a trimeric complex with CRM1 and RanGTP. However, RanBP3 does not bind to CRM1 like an export substrate. Instead, it can stabilize CRM1–export substrate interaction. Nuclear RanBP3 stimulates CRM1-dependent protein export in permeabilized cells. These data indicate that RanBP3 functions by a novel mechanism as a cofactor in recognition and export of certain CRM1 substrates. *In vitro*, RanBP3 binding to CRM1 affects the relative affinity of CRM1 for different substrates.

INTRODUCTION

The nuclear and cytoplasmic compartments of eukaryotic cells communicate with one another through nuclear pore complexes (NPCs) that span the nuclear envelope. Small ions, metabolites and small polypeptides can diffuse freely across NPCs, but most proteins and ribonucleoproteins (RNPs) are transported into or out of the nucleus by active, signal dependent, receptor-mediated processes (reviewed in Mattaj and Englmeier, 1998; Görlich and Kutay, 1999; Nakielnny and Dreyfuss, 1999).

Proteins related to the archetypal import receptor importin β function as transport receptors by binding cargo molecules, either directly or indirectly via adaptor proteins, and mediating their translocation through the NPC. The small GTPase Ran is a key regulator of receptor–transport substrate interaction. Ran is predicted to be predominantly in the GDP-bound form in the cytoplasm and in the GTP-bound form in the nucleus. Consequently, Ran can provide directionality to transport

processes (Görlich *et al.*, 1996b; Izaurralde *et al.*, 1997). Import complexes between importins and their cargoes form in the cytoplasm and are dissociated upon translocation to the nucleus when RanGTP binds to the importin (Rexach and Blobel, 1995; Görlich *et al.*, 1996b). Export complexes between exportins and their cargoes only form in the nucleus in the presence of RanGTP (Fornerod *et al.*, 1997; Kutay *et al.*, 1997, 1998; Arts *et al.*, 1998a) and are disassembled after translocation to the cytoplasm and GTP hydrolysis by Ran (Bischoff and Görlich, 1997; Kutay *et al.*, 1997). CRM1 (Fornerod *et al.*, 1997; Fukuda *et al.*, 1997; Stade *et al.*, 1997) exports numerous proteins that carry a leucine-rich nuclear export signal (NES) (Fischer *et al.*, 1995; Wen *et al.*, 1995) and also Snurportin1, which lacks a leucine-rich NES (Paraskeva *et al.*, 1999). CRM1 also mediates export of certain U snRNAs via the specialized adaptor protein PHAX (Ohno *et al.*, 2000). Thus, CRM1 recognizes diverse export signals on a variety of export substrates.

It is presently unclear whether CRM1's binding to this diversity of export substrates requires cofactors, but a possible candidate for such a function is the yeast protein Yrb2p. Yrb2p plays a role in protein export mediated by Xpo1p, the yeast homologue of CRM1 (Taura *et al.*, 1998; Noguchi *et al.*, 1999). Yeast cells mutant in the YRB2 gene, which encodes a nuclear protein with a RanBP1-like Ran-binding domain (RBD), show a defect in NES-mediated export. Overexpression of Yrb2p also inhibits CRM1-dependent nuclear export (Taura *et al.*, 1998; Noguchi *et al.*, 1999; Jones *et al.*, 2000). Yrb2p binds directly to Xpo1p (Taura *et al.*, 1998; Noguchi *et al.*, 1999), but how this interaction affects export is unclear.

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The nuclear protein RanBP3 is the human orthologue of Yrb2p (Müller *et al.*, 1998). This prompted us to investigate the role of RanBP3 in CRM1-mediated nuclear protein export in higher eukaryotes. We show that RanBP3 can stabilize the interaction between CRM1 and an export substrate and influence substrate choice by CRM1 *in vitro*. RanBP3 also stimulates CRM1-dependent NES protein export *in vitro*.

RESULTS

CRM1 is a major interaction partner of RanBP3

To characterize proteins that interact with human RanBP3, HeLa cell extract was bound to immobilized GST–RanBP3. Immobilized GST served as a negative control (Figure 1, lane 2). The bound fractions contained one major interaction partner of RanBP3 (lane 4). This was shown to be CRM1 by immunoblotting, whereas RanBP16, another transport receptor (Kutay *et al.*, 2000), did not bind to RanBP3 (Figure 1, bottom panel). An N-terminal fragment of RanBP3 (amino acids 1–304) also bound CRM1 specifically (lane 3), demonstrating that the RanBP1-like domain of RanBP3 is not required for this interaction (Müller *et al.*, 1998).

RanBP3 interaction with CRM1

RanBP3 could either interact with CRM1 similarly to export substrates or by a different mechanism. To address this point we measured the formation of trimeric CRM1–export substrate–RanGTP complexes. The assay measures protection of RanGTP from RanGAP-induced GTP hydrolysis on binding to an importin β -like transport receptor. For exportins, the Ran-bound GTP is only efficiently protected against RanGAP-induced hydrolysis when a complex between export receptor, RanGTP and export substrate is formed (Askjaer *et al.*, 1999; Paraskeva *et al.*, 1999). Preincubation of RanGTP with increasing concentrations of two export substrates, either BSA–NES (BSA conjugated with peptides of the HIV-1 Rev NES) or Snurportin1, and CRM1 resulted in progressive inhibition of RanGAP-induced GTP hydrolysis (Figure 2A, diamonds and triangles). Half-maximal inhibition was obtained at ~500 nM BSA–NES and 20 nM Snurportin1. RanBP3, in the presence of CRM1, also protected RanGTP against hydrolysis with half-maximal inhibition at 1 nM (Figure 2A, squares). RanBP3 alone did not inhibit GTP hydrolysis on Ran (data not shown). Thus, RanBP3 can form a high-affinity complex with RanGTP and CRM1.

Complexes between CRM1/RanGTP and export substrates are sensitive to leptomycin B (Fornerod *et al.*, 1997). RanBP3 formed a protective complex with RanGTP and CRM1 even in the presence of LMB (Figure 2B, closed squares), while Snurportin1 (Figure 2B, closed circles) and BSA–NES (data not shown; see Askjaer *et al.*, 1999) failed to form a protective complex with CRM1 and RanGTP when LMB was present.

RanBP1 catalytically dissociates export complexes by dissociating RanGTP and presenting it to RanGAP (Bischoff and Görlich, 1997; Kutay *et al.*, 1997; Askjaer *et al.*, 1999; Paraskeva *et al.*, 1999). In a GAP-protection experiment with RanGTP, CRM1 and Snurportin1, addition of RanBP1 quickly relieved the protection against RanGAP-mediated GTP hydrolysis (Figure 2C, closed circles). RanBP1 is also sufficient to quickly dissociate

Fig. 1. CRM1 is a RanBP3 binding partner. HeLa cell extract (lane 1) was added to either immobilized GST (lane 2) or truncated or full-length GST–RanBP3b (lanes 3 and 4 as indicated). Bound proteins were separated and detected by Coomassie staining (top panel) or immunoblotting (bottom panel).

other export receptor–RanGTP–export substrate complexes like CAS–RanGTP–importin α and Xpo-t–RanGTP–tRNA (Kutay *et al.*, 1997, 1998). In contrast, the complex between CRM1, RanGTP and RanBP3 was resistant to RanBP1-mediated dissociation (Figure 2C, closed and open squares). Thus, RanBP3 can form a tight complex with CRM1 and RanGTP that is resistant to RanBP1-mediated dissociation and that is LMB insensitive. We conclude that RanBP3 and the previously characterized export substrates bind to CRM1 differently. We found no evidence for significant interaction of RanBP3 with other importin β family members such as transportin, Xpo-t or importin β itself (Figure 1 and data not shown).

RanBP3 stabilizes the CRM1–export substrate interaction at an optimal concentration

We wanted to know whether the binding of export substrate by CRM1 was affected by RanBP3. An export substrate, GST fused to the HIV-1 NES (GST–NES) (Askjaer *et al.*, 1999), was immobilized and increasing amounts of RanBP3 were added to it with constant concentrations of CRM1 and RanGTP. CRM1 (at 1 μ M) and RanGTP (at 1.25 μ M) bind detectably to the export substrate when added together (Figure 3, lane 2). However, RanBP3 addition up to a certain concentration increased the amount of CRM1 and RanGTP bound to GST–NES, and RanBP3 also associated with the GST–NES affinity matrix under these conditions (Figure 3, lanes 3–5).

Fig. 2. A RanBP3–CRM1–RanGTP complex. **(A)** Fifty picomolar of Ran[γ - 32 P]GTP was incubated with 300 nM CRM1 and the indicated concentrations of BSA–NES (NES peptide CPVPLQLPPLRLTLD), Snurportin1 and RanBP3. After 30 min, 40 nM Rna1p was added. Hydrolysis of Ran-bound GTP was measured by 32 P-release after 30 s. **(B)** Thirty nanomolar CRM1 was incubated with 5 μ M leptomycin B or with buffer for 15 min then 100 pM Ran[γ - 32 P]GTP and RanBP3 or Snurportin1 were added as indicated. Thirty minutes later, GTPase activity was assayed. **(C)** Fifty picomolar of Ran[γ - 32 P]GTP was incubated for 30 min with 600 nM CRM1 and with 5 nM RanBP3 or 3 μ M Snurportin1. Six minutes after addition of 40 nM Rna1p, 25 nM RanBP1 was added as indicated. GTP hydrolysis by Ran was measured at the time points indicated.

Fig. 3. Biphasic effect of RanBP3 on export complex formation. Five micrograms of GST–NES (lanes 1–13) or GST–M10 (lanes 14–17) were immobilized then incubated with 1 μ M CRM1 (lanes 2–13 and 15–17), 1.25 μ M Ran(Q69L)GTP (lanes 2–7, 9–13 and 15–17) or Ran(Q69L)GDP (lane 8) and increasing amounts of RanBP3 (lanes 3–7, 8, 16 and 17) or RanBP3-wvaa (lanes 9–13). The highest concentration of added RanBP3 or RanBP3-wvaa was 2 μ M (lanes 7, 13 and 17); the molar ratio of RanBP3 and CRM1 is indicated. Bound proteins were analysed by SDS–PAGE.

A further increase in the RanBP3 concentration reduced, and finally prevented, binding of CRM1, RanGTP and RanBP3 to GST–NES (Figure 3, lanes 6 and 7). Thus, at optimal concentration, RanBP3 increased the interaction of CRM1 with an export substrate and RanGTP. The complexes formed in the presence of RanBP3 were specific to bona fide export substrates, as no binding was detected to the mutant GST–M10 substrate (lanes

14–17; see Fornerod *et al.*, 1997) or when RanGTP was substituted with RanGDP (Figure 3, lane 8). We conclude that RanBP3 works as a bifunctional protein in this assay; at low concentrations it stimulates export substrate binding by CRM1 and is part of a complex with CRM1, RanGTP and export substrate (see Supplementary data, available at *EMBO reports* Online), while at high concentration it prevents binding of CRM1 and RanGTP to export substrate.

In this four-component complex, RanGTP could be bound either by the importin β -like Ran binding N-terminus of CRM1, by the RBD of RanBP3, or by both simultaneously. To discriminate between these possibilities, we mutated residues predicted to be critical for the interaction with RanGTP (see Supplementary data). Both Glu352 and Arg353 in the RBD of RanBP3 were changed to alanines. This double mutant, RanBP3-wvaa, could be purified indistinguishably from wild-type RanBP3, indicating that the folding of the mutant protein was not grossly perturbed. However, RanBP3-wvaa bound RanGTP 200-fold less tightly than wild-type RanBP3 in the context of a trimeric RanGTP–CRM1–BP3 complex when tested in a RanGAP protection assay (data not shown). We added increasing concentrations of RanBP3-wvaa together with CRM1 and RanGTP to a GST–NES affinity matrix. RanBP3-wvaa, like RanBP3, increased the binding of CRM1 and RanGTP to the export substrate (Figure 3, lanes 9–11) but, unlike RanBP3, it did not inhibit the formation of the tetrameric complex at higher concentrations (lanes 12 and 13).

The two functions of RanBP3, stimulation of export substrate binding by CRM1 at low concentrations of RanBP3 and inhibition of CRM1–export substrate interaction at high concentrations, are

Fig. 4. Nuclear RanBP3 stimulates CRM1-dependent export. Fluorescein-labelled Nplc-M9-NES or Nplc-M9-M10 (200 nM) was imported in permeabilized HeLa cells for 15 min with 50 nM transportin, 60 nM importin β , Ran-mix and an energy-regenerating system with or without 50 nM Alexa594-labelled IBB-RanBP3b. The import mixtures were drained and control reactions fixed (top panels). The remaining samples were incubated for 15 min with or without 150 nM CRM1 in transport buffer, Ran-mix and energy as indicated, then fixed and analysed by confocal microscopy.

therefore separable. Although only wild-type RanBP3, which can bind RanGTP, inhibited export complex formation (Figure 3, lanes 6 and 7), we can exclude that the mechanism of inhibition is in competition with CRM1 for free RanGTP, since the addition of excess RanGTP did not change the concentration optimum for RanBP3 (data not shown). Furthermore, increasing CRM1 concentration did affect the RanBP3 concentration optimum (data not shown), suggesting that RanBP3 interacts with CRM1, resulting in complex stabilization.

Nuclear RanBP3 stimulates CRM1-dependent protein export

Our biochemical data show that an optimal concentration of RanBP3 stimulated the interaction between CRM1 and an NES protein. Since RanBP3 is a nuclear protein and export complex formation is a nuclear event, we asked whether nuclear RanBP3 could stimulate CRM1-dependent protein export in permeabilized human cells. RanBP3 was N-terminally tagged with the importin β -binding (IBB) domain of human importin α 1 (Görlich *et al.*, 1996a) to allow its efficient nuclear accumulation. Export of the shuttling protein Nplc-M9-NES (Englmeier *et al.*, 1999) was then compared in the presence and absence of RanBP3.

In a first step, Nplc-M9-NES was imported into the nuclei of permeabilized cells. In addition, importin β either alone or together with IBB-RanBP3 was added. This allowed import of IBB-RanBP3 in the indicated samples. After 15 min, Nplc-M9-NES had efficiently been imported into the nuclei with or without IBB-RanBP3 (Figure 4, top). IBB-RanBP3 was also efficiently imported (data not shown). The import reaction was drained from the coverslips and export was initiated by the addition of a limiting amount of CRM1, Ran Mix and an energy regenerating system. Weak export of Nplc-M9-NES occurred in the absence of added nuclear RanBP3, indicating that CRM1 and the components of the Ran Mix were sufficient for nuclear protein export of Nplc-M9-NES (Figure 4, bottom row, far left). However, export was further stimulated when nuclei were pre-loaded with RanBP3 (Figure 4, bottom, second from left). No export of Nplc-M9-NES was observed when CRM1 was omitted from the export mix (Figure 4, middle row). The export-deficient control substrate, Nplc-M9-M10, remained nuclear in both untreated and RanBP3-loaded nuclei (Figure 4, right panels).

RanBP3 can influence substrate choice by CRM1

To determine whether CRM1 binding to different export substrates is affected similarly by RanBP3, limiting amounts of

CRM1 and other exportins: similarities and differences

Fig. 5. RanBP3 affects CRM1 substrate interactions differentially. Fifty picomolar zz-CRM1 (100 μ l) was incubated with 100 pmol Ran(Q69L)GTP (lanes 3–6, 9 and 10), 100 pmol GST–NES (lanes 5–10) and 100 pmol Snurportin1 (lanes 1–4 and 7–10). In lanes 2, 4, 6, 8 and 10, 10 pmol RanBP3–wvaa were added. After incubation at 4°C for 30 min, 10 μ l IgG Sepharose were added and after 60 min at 4°C bound proteins were analysed by SDS–PAGE.

zz-tagged CRM1 were incubated in the presence or absence of RanBP3–wvaa and the binding of two competing substrates, Snurportin1 or GST–NES, analysed. Snurportin1 bound efficiently to zz-CRM1 in the presence of RanGTP (Figure 5, lane 3), and addition of RanBP3–wvaa slightly inhibited this binding (lane 4). GST–NES also bound to zz-CRM1 in the presence of RanGTP (lane 5), and under the conditions of this experiment addition of RanBP3–wvaa had no detectable effect on GST–NES binding to zz-CRM1 (lane 6). When Snurportin1 and GST–NES were allowed to compete for binding to zz-CRM1 in the presence of RanGTP, Snurportin1 bound efficiently, while GST–NES binding was greatly reduced (lane 9). This reflects the higher affinity of Snurportin1 compared to NES substrates for CRM1 (Paraskeva *et al.*, 1999; Figure 2A). In this competitive situation the addition of RanBP3–wvaa had opposing effects on Snurportin1 and GST–NES binding to zz-CRM1: Snurportin1 binding was decreased while binding of GST–NES was increased (Figure 5, lane 10). Snurportin1 and GST–NES binding to zz-CRM1 in the presence of RanBP3 were both RanGTP dependent (lanes 2 and 8). We conclude that RanBP3 leads to a discrimination between different classes of CRM1 export substrates. In the presence of RanBP3 the relative affinities of NES-containing substrates and Snurportin1 are altered.

DISCUSSION

We have studied the role of RanBP3 in CRM1-mediated nuclear protein export. RanBP3 stabilizes the interaction between CRM1, RanGTP and an export substrate at an optimal concentration. In transport assays in permeabilized cells, RanBP3 stimulates CRM1-mediated nuclear protein export. Our results indicate that RanBP3 functions to stimulate intranuclear recognition of some substrates by CRM1, and to increase the export of these substrates. In addition, RanBP3 influences substrate choice by CRM1 in *in vitro* binding assays.

A comparison of CRM1 with the export receptors CAS (Kutay *et al.*, 1997) and Xpo-t (Arts *et al.*, 1998a; Kutay *et al.*, 1998) reveals an interesting biochemical difference. Both CAS and Xpo-t bind their substrates with high affinity in the context of simple trimeric complexes: $K_{app} = 1$ nM for the CAS–importin α –RanGTP complex and 3 nM for an Xpo-t–tRNA–RanGTP complex. Stable trimeric complex formation is a prerequisite for efficient export of tRNA. Mutant tRNAs that display 100-fold lower binding than Xpo-t (a K_{app} of ~ 100 nM in the context of a trimeric Xpo-t–RanGTP–tRNA complex) are defective in nuclear export (Arts *et al.*, 1998b; Kutay *et al.*, 1998; Lipowsky *et al.*, 1999). In contrast, the interactions between CRM1, RanGTP and most export substrates containing a leucine-rich NES tested *in vitro* are weak. Rev and BSA–NES, for example, bind to CRM1 and RanGTP with an apparent K_d of ~ 500 nM (Askjaer *et al.*, 1999; Paraskeva *et al.*, 1999). Surprisingly, despite their low apparent affinity for CRM1 *in vitro*, these proteins are rapidly and efficiently exported *in vivo* (Fischer *et al.*, 1995). If one assumes that formation of stable export complexes is also a prerequisite for CRM1-mediated protein export, then one would have to postulate a mechanism for increasing the affinity of substrate interaction with CRM1. RanBP3 exhibits the biochemical and functional properties required for this function.

RanBP3 and substrate recognition *in vitro*

We show that RanBP3 participates in substrate recognition by CRM1 *in vitro*. At an optimal concentration, RanBP3 increases the stability of the CRM1–RanGTP–export substrate interaction. Therefore, the stimulatory function of RanBP3 is explicable if we assume that RanBP3 stabilizes CRM1–RanGTP complexes in a conformation competent for export substrate, i.e. NES protein binding. Subsequent binding of export substrates to CRM1–RanGTP complexes preformed on RanBP3 should then be facilitated when compared with formation of the trimeric CRM1–RanGTP–export substrate complex from the free components.

A second feature of RanBP3 is its ability to inhibit export complex formation at higher concentrations. This inhibitory function of RanBP3 is dependent on a functional RBD in RanBP3 but it is nevertheless not readily explained (Figure 3). One possible model is that the RBD of RanBP3 may have a significant affinity for RanGTP in the appropriate context, for example, when RanGTP is part of a trimeric CRM1 export complex. A first molecule of RanBP3 would bind to a CRM1–RanGTP–NES complex, through CRM1, and stabilize it, but if RanBP3 is present in sufficient quantity, a second molecule might remove RanGTP from CRM1. This would force CRM1 back into a non-Ran-bound, non-substrate-binding import conformation. Support for this model comes from the ability of RanBP3–wvaa, whose interaction with RanGTP is compromised, to stabilize but not to destabilize a CRM1 export complex.

In the presence of RanBP3, the otherwise low affinity CRM1–RanGTP substrate GST–NES outcompetes the otherwise high affinity substrate Snurportin1. This suggests that CRM1, when bound to RanBP3, is in a conformation that favours binding of leucine-rich NES-containing substrates over binding of other export substrates like Snurportin1. The three available crystal structures of

importin β provide evidence for a large conformational repertoire available to this protein (Cingolani *et al.*, 1999; Vetter *et al.*, 1999; Bayliss *et al.*, 2000), and it is likely that conformational flexibility is a general feature of nuclear transport receptors.

The role of RanBP3 in substrate recognition *in vivo*

Our data suggest that proteins with a leucine-rich NES can initially be recognized *in vivo* as part of a RanBP3–CRM1–export substrate–RanGTP complex. This model predicts that without RanBP3, CRM1 should export substrates with low affinity leucine-rich NESs less efficiently, since recognition of these substrates is predicted to be impaired without RanBP3. This is observed in our *in vitro* export assays. Our biochemical data also predict that too much RanBP3 *in vivo* would be inhibitory to NES–protein export. Indeed, injection of exogenous RanBP3 into *Xenopus* oocyte nuclei selectively inhibited CRM1-dependent NES-mediated export, but not other export pathways tested (see Supplementary data).

Previous genetic experiments from *Saccharomyces cerevisiae* provide additional data on RanBP3 activity. In a yrb2 deletion strain, Xpo1p-mediated protein export is inefficient. Restoration of Yrb2p levels to normal restores export, while Yrb2 over-expression selectively inhibits Xpo1p-mediated export (Taura *et al.*, 1998; Noguchi *et al.*, 1999). The selectivity of this effect for Xpo1p substrates argues against a model where Yrb2p at higher concentrations would inhibit export by simply titrating out nuclear RanGTP, and the effects are consistent with, and explicable by, the biochemical data presented here. While this manuscript was under revision, data complementary to those presented were published elsewhere (Lindsay *et al.*, 2001).

METHODS

Plasmids and protein expression in *Escherichia coli*. CRM1, Nplc-M9-NES, Nplc-M9-M10 (Englmeier *et al.*, 1999), zz-CRM1 (Askjaer *et al.*, 1999), RanQ69L (Izaurrealde *et al.*, 1997), GST–RanBP3b, Rna1p (Bischoff *et al.*, 1995) and His₆-tagged RanBP3b (Müller *et al.*, 1998) were expressed and purified as previously described.

Purification of RanBP3-binding proteins. Preparation of HeLa cell extract and affinity selection have been previously described (Kutay *et al.*, 2000). Purification of proteins interacting with GST–RanBP3 is described in the Supplementary data.

RanGAP protection and solution binding assays. GTPase assays were carried out as previously described (Bischoff *et al.*, 1995). Exact conditions for solution binding assays are given in the Supplementary data.

Permeabilized cell assay. *In vitro* nuclear transport assays were as described (Kutay *et al.*, 1997), with details in the Supplementary data.

Supplementary data. Supplementary data are available at *EMBO reports* Online.

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L. Englmeier et al.

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