RanBP1 is crucial for the release of RanGTP from importin β-related nuclear transport factors

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Abstract Nucleocytoplasmic transport appears mediated by shuttling transport receptors that bind RanGTP as a means to regulate interactions with their cargoes. The receptor·RanGTP complexes are kinetically very stable with nucleotide exchange and GTP hydrolysis being blocked, predicting that a specific disassembly mechanism exists. Here we show in three cases receptor·RanGTP·RanBP1 complexes to be the key disassembly intermediates, where RanBP1 stimulates the off-rate at the receptor/RanGTP interface by more than two orders of magnitude. The transiently released RanGTP·RanBP1 complex is then induced by RanGAP to hydrolyse GTP, preventing the receptor to rebind RanGTP. The efficient release of importin β from RanGTP requires importin α, in addition to RanBP1.

Key words: Nuclear transport; Importin; Transportin; CAS; RanBP1

1. Introduction

Transport of macromolecules between the cell nucleus and the cytoplasm occurs through the nuclear pore complexes (NPCs) and is generally an energy-dependent and signal-dependent process. These transport signals are thought to be recognised by specific transport receptors that shuttle continuously between the two compartments (for review, see [1–4]). The transport receptors from higher eukaryotes identified to date include the importin α/β heterodimer where importin α recognises the classical nuclear localisation signal (NLS) and importin β in turn accounts for the actual translocation through the NPC and carries importin α with the NLS substrate into the nucleus (for review, see [1,5,6]). CAS is the export factor that returns importin α back to the cytoplasm [7]. Transportin is the import factor for proteins with an M9 import signal [8–10], and CRM1 accomplishes nuclear export of proteins with a leucine-rich nuclear export signal (NES) [11,12].

In addition to these pathway-specific transport factors, the small GTPase Ran appears to be essential for most nucleocytoplasmic transport routes as has been demonstrated for the import of NLS- and M9-containing proteins as well as for import of snRNPs [9,13–16] and the export of importin α [7,15,17], NES proteins [15,18], tRNA [15], U snRNA, and that of several mRNAs [15,19,20].

Ran's nucleotide-bound state is controlled by the nucleotide exchange factor RCC1 which can charge Ran with GTP [21], the GTPase activating protein RanGAP1 which converts RanGTP into RanGDP [22–25], and by RanBP1 whose binding to RanGTP further stimulates the GTPase activation by RanGAP1 [26–28]. Since RCC1 is an exclusively nuclear protein [29], RanGTP is most likely generated only in the nucleus. RanGAP1 and its co-activator RanBP1 have the opposite localisation, they are excluded from the nuclei and deplete RanGTP from the cytoplasm [30–34]. This asymmetric distribution of RCC1, RanGAP1, and RanBP1 should result in a steep concentration gradient across the NPC with a very low cytoplasmic RanGTP concentration and high levels in the nucleus. We have previously suggested [1,35] that this RanGTP concentration gradient is crucial for the directionality of nucleocytoplasmic transport, ensuring that shuttling transport receptors carry their cargoes in one direction only.

The transport factors importin β, transportin, CAS and CRM1 belong to a superfamily of RanGTP binding proteins [36,37]. These Ran binding proteins are distinct from the RanBP1 type. The two classes bind to distinct sites on Ran [35,38–40], their Ran binding motifs are unrelated in sequence, and whereas the activation of the RanGTPase is stimulated by binding of RanBP1 to RanGTP [27,28], it is blocked by binding of an importin β-related factor [7,35,37,40–42]. The interaction of RanGTP with importin β-like transport factors regulates substrate binding in a compartment-specific manner: The import factors importin β and transportin bind their substrates only in the absence of RanGTP [15,35,38,43], i.e. in the cytoplasm, and release it upon interaction with RanGTP which should happen in the nucleus where the RanGTP concentration is predicted to be high [35]. Importin β and transportin are probably exported to the cytoplasm as RanGTP complexes [15]. This should preclude re-export of the cargoes they just carried in. Before the next substrate molecule can be bound and imported from the cytoplasm, the import factors need to be released from RanGTP.

Substrate binding to the exportins (CRM1, CAS) appears to be regulated in exactly the opposite way, in being greatly enhanced by simultaneous RanGTP binding [7,12]. This should happen in the nucleus where the RanGTP concentration is predicted to be high. The trimeric substrate·exportin·RanGTP complex is then transferred to the cytoplasm. There, RanGTP needs to dissociate from the complex to allow the exportin to release the substrate, to re-enter the nucleus and to bind and export the next cargo molecule.

The disassembly of RanGTP complexes with importin β, transportin, and CAS is thus an essential part of both nuclear import and export cycles. However, the complexes are kinetically very stable and resist GTPase activation of Ran by RanGAP. We investigated the mechanism of the disassembly re-

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Abbreviations: RanGTP, GTP-bound form of Ran; RanGDP, GDP-bound form of Ran; GAP, GTPase activating protein; NPC, nuclear pore complex

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actions and identified RanBP1 as a key player in the process that directly promotes dissociation of RanGTP from the transport factors. Ran appears to be transiently released as a RanGTP·RanBP1 complex which then can irreversibly be converted by RanGAP1 into RanGDP plus RanBP1. RanBP1 can participate in another round of disassembly. RanGAP1 and RanBP1 therefore act catalytically in the process.

2. Materials and methods

2.1. Protein expression and purification

The expression and purification of the following proteins was as described previously: recombinant human Ran [44], RCC1 [45], *S. pombe* Rna1p [23], murine RanBP1 [27], human His-tagged importin α (Rch1), human His-tagged importin β [35], His-tagged transportin, z-fusion of transportin [15], *N*-His CAS and z-tagged Ran [7].

2.2. Enzymatic assays

Labeling of Ran with [γ-^{32}P]GTP or [α-^{32}P]GDP, GTPase and guanine nucleotide exchange assays were performed as described [22,27,35]. The concentration of Ran[γ-^{32}P]GTP was adjusted by addition of unlabeled RanGTP.

To determine the RanBP1-induced dissociation of RanGTP from transportin, 2 μg purified rabbit IgG per dot were applied via vacuum filtration onto nitrocellulose (0.45 μm, Schleicher and Schuell). Then the 3 mm dots were cut out and transferred to 1.5 ml reaction tubes and blocked for 30 min with 50 μl of 1% hydrolysed gelatine in TBS. Two μl of 26 μM z-transportin was added to each tube and allowed to bind for 15 min to the immobilised IgG (note 'z' is the IgG binding domain from protein A). After two washes with assay buffer (20 mM HEPES-NaOH, pH 7.4, 100 mM NaCl, 1 mM $MgCl_2$, 1 mM 2-mercaptoethanol, 0.02% sodium azide, 0.05% hydrolysed gelatine) 30 μl of 100 nM Ran[γ-^{32}P]GTP was added per tube. After 15 min incubation, unbound Ran was washed away and 50 μl of 1 μM His-tagged transportin or a mixture of 0.1 μM RanBP1 and 1 μM transportin were added to the samples. After the time intervals indicated in Fig. 3A, the supernatant was recovered and filters were washed once with assay buffer. The radioactivity of the filters and supernatants was determined separately. Values for the supernatant are corrected for intrinsic GTP hydrolysis on Ran.

3. Results

3.1. Transportin, like importin β, blocks the activation of the RanGTPase

When RanGTP is complexed with importin β or a related factor, its GTPase activation by RanGAP is blocked. The dose-dependence of this effect is exemplified for importin β and transportin in Fig. 1A. The concentration required for half-maximum inhibition gives an estimate for the dissociation constants (K_D) of 0.5 nM for importin β·RanGTP and 1 nM for the transportin·RanGTP complex. Both complexes are kinetically very stable with half-lifes of approximately 4 h in the case of importin β·RanGTP and 2.5 h for transportin·RanGTP (see below). These complexes therefore would constitute nearly irreversible sinks for the involved transport factors, unless some efficient mechanism for their rapid disassembly would exist.

Fig. 1B shows that the addition of even sub-nanomolar concentrations of RanBP1 efficiently releases the GTPase block caused by binding of transportin to RanGTP. The half-maximum effect was observed at about 0.3 nM RanBP1 which is close to the reported K_D of the RanGTP·RanBP1 complex [35,46,47]. To obtain this number we had to use a RanGTP concentration (50 pM) far below that of RanBP1. We next wanted to know if RanBP1 could initiate multiple rounds of disassembly and therefore measured this reaction at a much higher concentration of the transportin·RanGTP complex (100 nM). The time-course in Fig. 1C shows that even 1 nM RanBP1 greatly stimulated the GTPase activation, and for example after 10 min each molecule of RanBP1 had participated in average in 50 rounds of disassembly. This can only be explained if RanBP1 acts catalytically in the process.

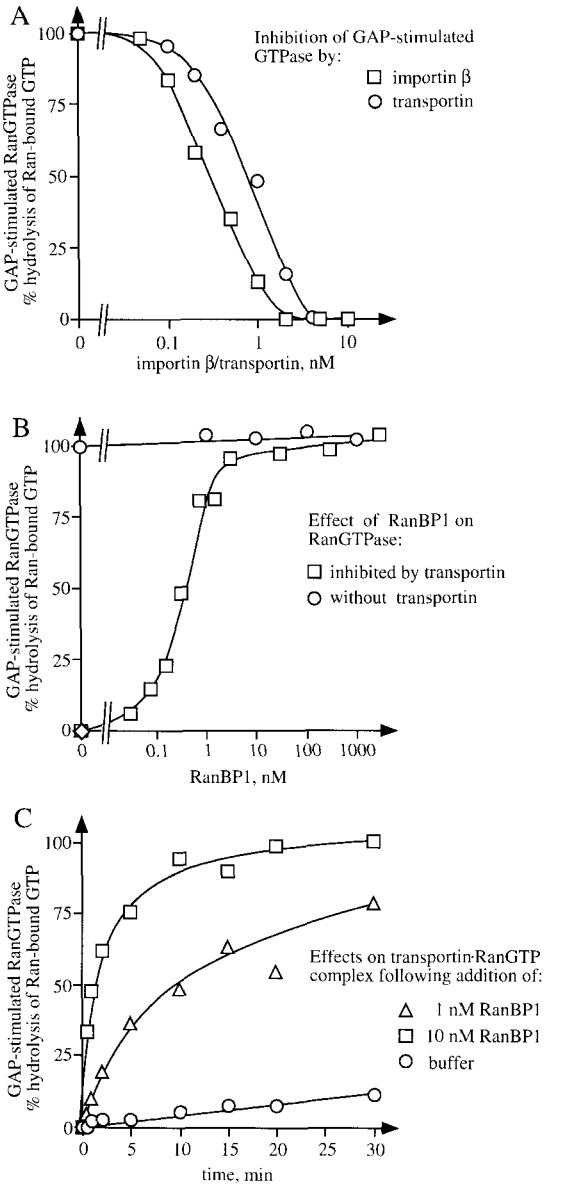

Fig. 1. A: Importin β or transportin prevent the GAP-dependent activation of the RanGTPase. Fifty pM Ran[γ-^{32}P]GTP was pre-incubated with indicated concentrations of either importin β, transportin, or incubation buffer. After 20 min, 20 nM RanGAP (Rna1p from *S. pombe*) was added and 2 min later the hydrolysis of Ran-bound GTP was determined as released [^{32}P]phosphate. B: RanBP1 relieves the GAP resistance of the transportin RanGTP complex. 50 pM Ran[γ-^{32}P]GTP was pre-incubated for 20 min with 10 nM transportin or with buffer as a control. Then 20 nM RanGAP was added followed by the addition of RanBP1 to the indicated final concentrations. After 2 min, hydrolysis of Ran-bound GTP was determined as released [^{32}P]phosphate. C: RanBP1 acts catalytically in relieving the GAP resistance of the transportin·RanGTP complex. 100 nM Ran[γ-^{32}P]GTP was pre-incubated with 150 nM transportin to form a transportin·RanGTP complex. Then 20 nM RanGAP and either buffer, 1 nM or 10 nM RanBP1 was added and GTP hydrolysis was measured at indicated time points.

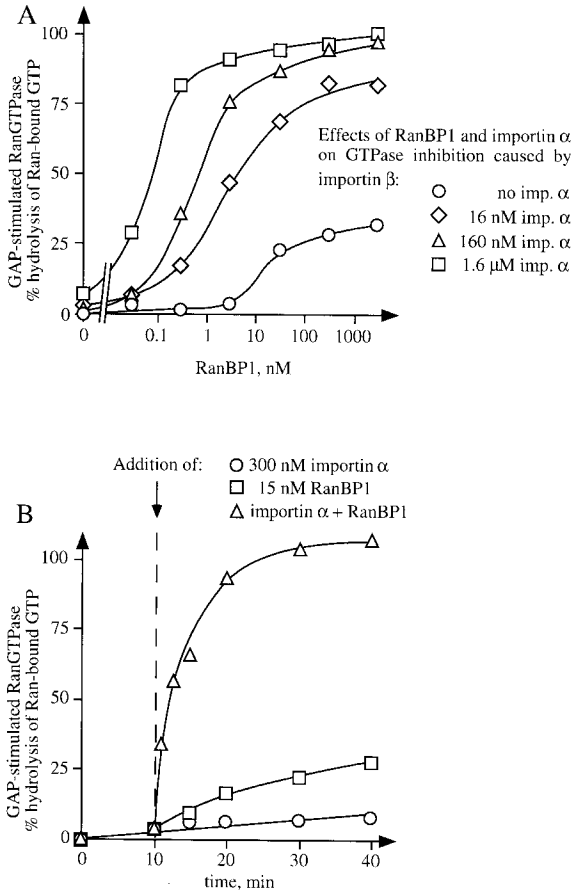

Fig. 2. A: Importin α and RanBP1 co-operatively relieve the GAP resistance of the importin β·RanGTP complex. 50 pM Ran[γ-^{32}P]GTP was pre-incubated for 20 min with 15 nM importin β and importin α at indicated final concentrations. Then 20 nM Rna1p was added, followed by addition of RanBP1 to the indicated final concentrations. After 2 min, hydrolysis of Ran-bound GTP was determined as released [^{32}P]phosphate. B: Panel shows a time-course of the effects described in panel A. 10 nM Ran[γ-^{32}P]GTP was pre-incubated for 30 min with 30 nM importin β in a volume of 500 μl. After 30 min, the initial reaction was started by the addition of 100 nM RanGAP and at indicated time points, GTP hydrolysis was determined in 50 μl aliquots. After 10 min, importin α and RanBP1 were added to the indicated final concentrations.

It should be noted that the expected disassembly intermediate, the transportin·RanGTP·RanBP1 complex can indeed be isolated (not shown). For two importin β-like proteins, namely RanBP5 and RanBP7, analogous complexes have previously been reported [37,40].

The GAP resistance of the transportin·RanGTP complex and its sensitisation towards GTPase activation by RanBP1 are thus properties shared with the analogous RanGTP complexes of RanBP5, RanBP7, Yrb4p, Pse1p, and also the importin α·CAS·RanGTP complex [7,37,40,42].

3.2. *RanBP1 and importin α are together required to efficiently disassemble the importin β·RanGTP complex*

In contrast to all other importin β-like transport factors tested so far, the GTPase block on importin β-bound RanGTP is not efficiently removed by RanBP1 alone ([35,40,42,48,49] and see Fig. 2A). We therefore searched for an additional activity required for efficient release and found importin α to be the missing factor (Fig. 2A), which agrees with a recent report by Floer et al. (1997). The addition of importin α alone, even at micromolar concentrations, had little effect on the release of Ran's GTPase block (see Fig. 2A and B). However, when RanBP1 was also added, a dramatic stimulation of Rna1p-induced GTP hydrolysis was evident. At saturating concentrations of importin α (1.6 μM which is comparable to the cellular concentration) the concen-

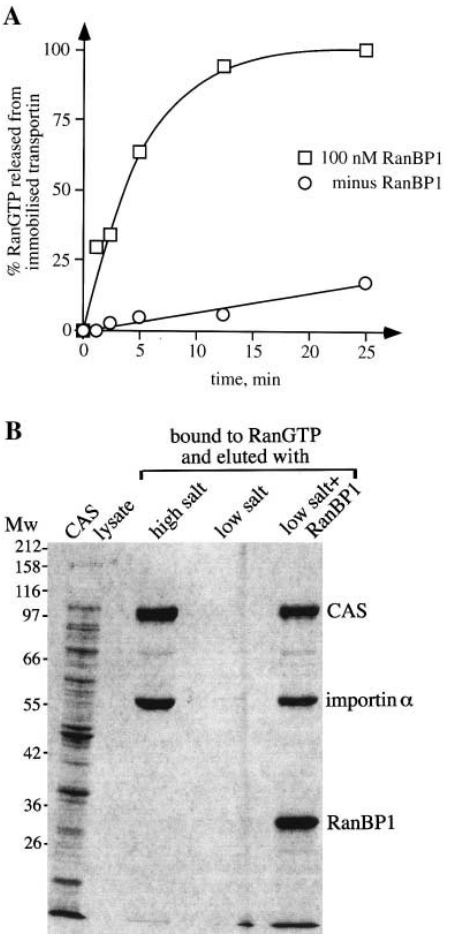

Fig. 3. A: RanBP1 directly induces disassembly of the transportin·RanGTP and the importin α·CAS·RanGTP complexes. Transportin, tagged with the IgG binding z-domain from protein A, was bound to IgG-nitrocellulose (for details of the assay see Section 2). Then, Ran[γ-^{32}P]GTP was allowed for 15 min to bind the immobilised transportin and non-bound radioactivity was washed away. Then, excess of non-immobilised transportin (i.e. lacking the z-tag) was added as a trap for RanGTP released from immobilised transportin. Then buffer or 100 nM RanBP1 was added and after the indicated time intervals, the radioactivity was determined on the filter and in the supernatant. Dissociation of RanGTP from transportin is given as the ratio between filter-bound and free radioactivity. B: CAS was expressed in *E. coli*, a lysate was prepared and 0.5 μM importin α was added. Three ml of the mixture (corresponding to 30 ml bacterial culture) was bound to z-tagged RanGTP that had been immobilised to 30 μl IgG Sepharose. The Sepharose was washed 5 times with 1 ml low salt binding buffer (containing 0.1 M NaCl) and split into three. Elution was performed with 80 μl of either high salt buffer (1 M NaCl), low salt buffer (0.1 M salt), or with low salt buffer plus 1 nmole RanBP1. The figure shows the Coomassie stained gel loaded with 2 μl of the lysate and 1/10 of the eluates. Note that CAS and importin α could be eluted from RanGTP in the absence of RanBP1 only at high salt concentration. In contrast, in the presence of RanBP1 this disassembly also occurred at physiological ionic strength. z-tagged Ran remained bound to the IgG Sepharose at low or high salt.

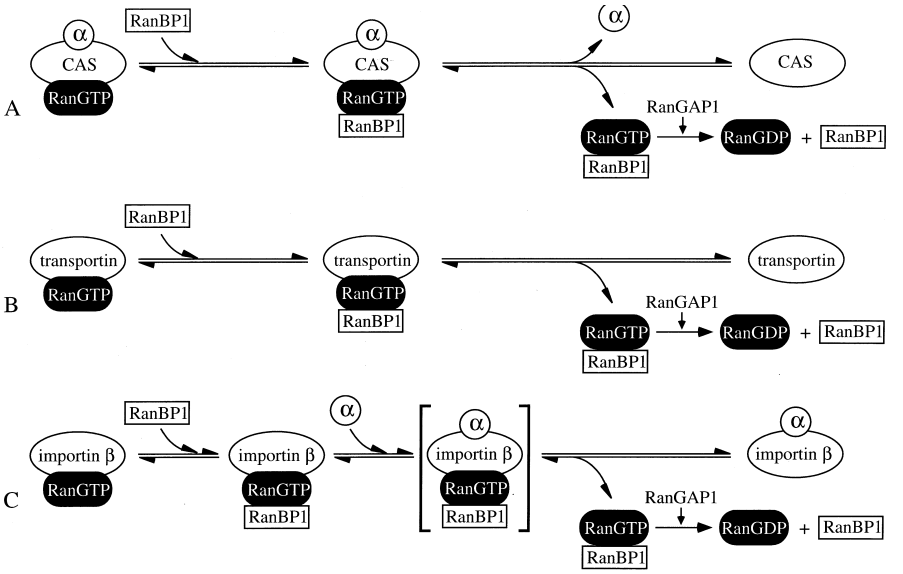

Fig. 4. Model for the disassembly of transport factor·RanGTP complexes. Disassembly of complexes of Ran·GTP and A: CAS; B: transportin; and C: importin-β. For details see main text.

tration of RanBP1 required for 50% release was shifted to the sub-nanomolar range (Fig. 2A).

We next examined GTP hydrolysis by Ran in time-dependent measurements. To 10 nM of RanGTP, a 3-fold molar excess of importin β was added. Then the GTPase reaction was started by addition of Rna1p (RanGAP). The high concentration of 100 nM Rna1p ensures that once RanGTP is dissociated from importin β, its GTPase is immediately activated before RanGTP can rebind to importin β. This allows to calculate the half-life of the importin β·RanGTP complex by measuring GTP hydrolysis on Ran. From Fig. 2A and B we estimate a half-life of 4 h for the importin β·RanGTP complex. The presence of importin α alone had basically no influence on the stability. If RanBP1 alone was added, only a slight increase in GTPase activation was observed (Fig. 2B). However, the addition of both, importin α (300 nM) and RanBP1 (15 nM) resulted in a roughly 200-fold stimulation of the RanGTPase (more than 700-fold at 1 μM importin α and 1 μM RanBP1, not shown).

3.3. *RanBP1 directly stimulates dissociation of RanGTP from transportin and the CAS·importin α complex*

We next wanted to know if RanBP1 could destabilise transport factor·RanGTP complexes also in the absence of RanGAP. To measure the dissociation of RanGTP from e.g. immobilised transportin directly, re-binding needs to be prevented. This was achieved by addition of an excess of non-immobilised transportin for competition. The half-life of the RanGTP transportin complex determined by this method is approximately 2 h (see Fig. 3A, curve 'minus RanBP1') which is in good agreement with the number obtained in Fig. 1C. Strikingly, when RanBP1 was added, the dissociation of RanGTP from transportin was accelerated by more than 100-fold.

We have recently shown, that importin α is exported from the nucleus as an importin α·CAS·RanGTP complex which can be disassembled by catalytic amounts of RanBP1 plus RanGAP1 [7]. To show that also in this case RanBP1 is the primary dissociating agent, we performed the experiment shown in Fig. 3B: CAS was expressed in *E. coli*, a lysate was prepared, supplemented with importin α, and applied to immobilised RanGTP, where the trimeric importin α·CAS·RanGTP complex assembled. As seen from the figure, CAS and importin α could be eluted at high ionic strength as reported before, but remained on the column at 100 mM NaCl (low salt). If the low salt buffer, however, also contained RanBP1 in slight molar excess over Ran, then CAS and importin α were efficiently released, even in the absence of RanGAP. Since importin β-like factors, including CAS and transportin, share related RanGTP binding motifs and behave similarly in the kinetic assays, it appears likely that RanBP1 directly promotes dissociation of all these factors from RanGTP. The dissociation is made irreversible by triggering GTP hydrolysis in the presence of RanGAP1.

4. Discussion

Nucleocytoplasmic transport of most classes of macromolecules directly depends on the GTPase Ran and appears to be mediated by shuttling transport receptors that bind RanGTP as a means to regulate the interaction with their cargoes. Candidate transport receptors are the constituents of a superfamily of Ran binding proteins that include roughly a dozen factors in yeast and probably even more in higher eukaryotes [36,37]. In vertebrates, the proven transport factors are the import receptors importin β and transportin, and the exportins CAS and CRM1 (see Section 1). To accomplish multiple rounds of transport, these factors have to shuttle continuously between nucleus and cytoplasm. Binding of RanGTP in the nucleus releases the import substrates from the import receptors whereas it stabilises the cargo binding to the exportins. After export of the resulting complexes to the cytoplasm, RanGTP needs to be released from the transport receptors in order to unload the cargoes from exportins and to allow the import factor to bind and import the next substrate molecule.

Here, we have investigated the mechanisms by which RanGTP is released from either importin β, transportin, or

CAS. These transport factor·RanGTP complexes are kinetically very stable and resistant towards GTPase activation by RanGAP1. Our data suggest that it is the Ran binding protein RanBP1 that promotes the initial dissociation of Ran from the transport factors. Ran is transiently released as a RanGTP·RanBP1 complex which is an optimal substrate for GTPase activation by RanGAP. GTP hydrolysis renders the dissociation irreversible since the resulting RanGDP has no significant affinity for either the transport factors or RanBP1. RanBP1 can therefore participate in another round of disassembly and acts catalytically in the process, similar to RanGAP. From our data, we suggest the disassembly mechanisms as depicted in Fig. 4. Crucial for this process is that RanBP1 and the respective importin β-like factor can simultaneously bind to RanGTP because their binding sites on Ran are distinct [37–40,50]. In the case of the importin α·CAS·RanGTP complex, the binding of RanBP1 to RanGTP greatly weakens the interaction between CAS and RanGTP and is sufficient for efficient release of Ran. This would also explain why injection of RanBP1 into the nucleus completely blocks export of importin α from the nucleus [15]. The disassembly of the transportin·RanGTP complex appears very similar to that of the importin α·CAS·RanGTP complex. A quantitative difference is that the transportin·RanGTP·RanBP1 complex can be isolated and appears stable in a thermodynamic sense. The main effect of RanBP1 appears to be a kinetic destabilisation and we found at least a 200-fold increased off-rate at the transportin/RanGTP interface.

In the case of the importin β·RanGTP complex, efficient disassembly requires importin α in addition to RanBP1. Because we have used a saturating concentration of RanGAP in these assays, the GTPase activation on the RanGTP·RanBP1 complex cannot have been rate-limiting. Therefore, importin α must have increased the rate by which RanGTP dissociates from importin β. This can only be explained by a physical interaction with importin α and we suggest a tetrameric importin α·importin β·RanGTP·RanBP1 complex as a transient intermediate. Such an intermediate could formally exist because the binding sites for Ran and importin α on importin β do not overlap [50,51]. The proposed shortlived tetramer could either release importin α again, or decay into two complexes, namely RanGTP·RanBP1 and the importin α/β heterodimer. The RanGTP·RanBP1 complex would be very efficiently removed from the equilibrium by GTPase activation. The released importin α/β heterodimer would be free for the next round of nuclear import. Importin β very likely can afford this more complicated disassembly mechanism because the cytoplasm contains micromolar concentrations of importin α. Since importin α can be regarded as the import substrate for importin β we also tested the effect of transportin's import substrate on the dissociation of the transportin·RanGTP complex. However we found no significant acceleration of the RanBP1-induced disassembly in the presence of the M9 domain (not shown).

Two related studies have also investigated the disassembly mechanism of the importin β·RanGTP complex. Lounsbury et al. (1997) suggested that RanBP1 and RanGAP1 alone are sufficient for the process. Subsequently, this view was weakened when the authors noticed they had used a defective importin β clone with a 10-fold decreased affinity for Ran. Despite the technical difficulty, the experiment emphasises that RanBP1 is the key-player in the disassembly.

Another study by Floer et al. (1997), looking at the yeast importin β·RanGTP complex, suggested that importin α alone would disassemble the complex. In their model, RanBP1 would bind to the already released RanGTP and aid RanGAP1 to trigger GTP hydrolysis. If this model was correct, then a further increase in the RanGAP concentration should have the same effect as addition of RanBP1. In our hands, this is certainly not the case. RanBP1 stimulates the disassembly more than a 200-fold even in the presence of micromolar concentrations of RanGAP.

Consistent with a role in the cytoplasmic disassembly of transport factor·RanGTP complexes, RanBP1 and RanGAP1 are excluded from the nucleus. The yeast homologues Yrb1p and Rna1p are encoded by essential genes and are both required for nucleocytoplasmic transport [25,52,53]. RanBP1 was found to stimulate protein import into nuclei of permeabilised mammalian cells when importin was present at a limiting concentration, i.e. when import was dependent on factor recycling [38].

The nuclear pore protein RanBP2, identified in higher eukaryotes contains four domains that are functionally equivalent to RanBP1 [39,54,55]. RanBP2 is located at the cytoplasmic filaments of the nuclear pore complex and forms a tight complex with a sub-population of RanGAP1 that is covalently modified with the ubiquitin-related SUMO-1 [32,33]. This RanBP2·RanGAP1 complex should be particularly efficient in releasing RanGTP from transport receptors and we suggest that this might be the primary function of this complex.

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