GTP Hydrolysis Links Initiation and Termination of Nuclear Import on the Nucleoporin Nup358*

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Binding of GTP-bound Ran (RanGTP) to karyopherin β1 (Kapβ1) releases import cargo into the nucleus. Using an ultrastructural, biochemical, and functional approach, we have studied the mechanism by which Kapβ1-RanGTP is recycled at the nuclear pore complex for repeated rounds of import. In vitro, Kapβ1 bound to the RanBP1-homologous (RBH) domains of Nup358 in the presence of either RanGTP or RanGDP, forming trimeric complexes. The Kapβ1-RanGTP-RBH complex resisted dissociation by RanBP1 and GTP hydrolysis by Ran GTPase activating protein 1. Ran-dependent binding of gold-conjugated Kapβ1 to the cytoplasmic fibers of the nuclear pore complex in digitonin-permeabilized cells and RanBP1 competition confirmed the in vitro binding data. Interaction of karyopherin α and a classical nuclear localization sequence peptide with the Kapβ1-RanGTP-RBH complex stimulated GTP hydrolysis by Ran GTPase activating protein 1 both in vitro and in permeabilized cells. This GTP hydrolysis was required for reinitiation of import of a nuclear localization sequence-bearing substrate in permeabilized cells. These data suggest that GTP hydrolysis on the RBH domains of Nup358 couples the termination of one cycle of nuclear import with the initiation of the next.

Nuclear import of proteins bearing a classical nuclear localization sequence (NLS) has been recently reviewed in Refs. 1–3. In digitonin-permeabilized cells, nuclear import of NLS-bearing proteins is mediated by a karyopherin α-karyopherin β1 (Kapα-Kapβ1) heterodimer. Kapα and Kapβ1 are also called importins α and β, respectively. Kapα binds to the NLS, whereas Kapβ1 interacts with the nuclear pore complex (NPC), resulting in docking of the cargo at the NPC. Other soluble factors including the small GTPase Ran, p10 (also called NTF2), and the small Ran-binding protein RanBP1 help to translocate the cargo-carrier complex through the NPC and release the cargo inside the nucleus in the presence of free GTP. The NPC is a large complex of proteins (nucleoporins) that spans the nuclear membrane and has filamentous projections into both the nucleus and cytoplasm (3–6). Although several nucleoporins interact with Kapβ1 in vitro, the exact sequence of interactions between Kapβ1, nucleoporins, and other soluble factors that result in translocation from the cytoplasmic to the nuclear side of the NPC and back is not well understood.

Ran binds to guanine nucleotides and cycles between a GDP-bound form (RanGDP) and a GTP-bound form (RanGTP). Nuclear transport is thought to require interconversion between these two forms (7–9). The interconversion of RanGTP and RanGDP is regulated by two factors. Ran GTPase activating protein 1 (RanGAP1), located predominantly in the cytosol and the cytoplasmic surface of the NPC, activates RanGTPase activity and thus converts RanGTP to RanGDP. RanGAP1 is targeted to the NPC by covalent modification with a small ubiquitin-like molecule (SUMO-1) (10, 11). SUMO-RanGAP1 is attached to the C terminus of Nup358 and is the only species of RanGAP1 retained in digitonin-permeabilized cells (10, 12, 13). RCC1, a predominantly intranuclear guanine nucleotide exchange factor for Ran, allows the release of bound nucleotide and reloading of Ran (7, 8, 14–16). The cellular concentration of GTP is higher than that of GDP; therefore, RCC1 is thought to favor the formation of RanGTP. The cellular distribution of these two factors suggests that nuclear Ran is predominantly in the GTP-bound form, whereas cytoplasmic Ran is mostly GDP-bound.

RanGTP binds to Kapβ1 leading to the release of Kapα along with the bound cargo into the nucleus (17–19). The Kapβ1-RanGTP complex then travels back through the NPC (20) presumably to initiate another cycle of import. Binding of Kapβ1 to RanGTP inhibits RanGAP1-mediated GTP hydrolysis (21). Yet nuclear import is favored when Ran is provided in its GDP bound form and is inhibited by nonhydrolyzable analogs of GTP and mutants of Ran that do not hydrolyze GTP (19, 22–28). The site and mechanism by which GTP hydrolysis occurs after formation of the Kapβ1-RanGTP complex are not well understood. RanBP1 binds strongly to RanGTP (29–31) and weakly to RanGDP (32, 33) and forms trimeric complexes with Kapβ1 and either RanGDP or RanGTP (34, 35). In vitro studies with yeast proteins have shown that RanBP1 in concert with Kapα and a C-terminal portion of the nucleoporin Nup1p can reverse the inhibition of RanGAP1 activity imposed by binding of RanGTP to Kapβ1 (36). Similar results have been reported with mammalian factors (37). However, it is not clear whether and where these reactions occur in cells. Recent studies provide evidence that GTP hydrolysis is not required for translocation of cargo through the NPC (38–40). This leaves open the possibility that GTP hydrolysis is required for recycling of the Kapβ1-RanGTP complex for repeated rounds of import.

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¶ The abbreviations used are: NLS, nuclear localization signal; Kap, karyopherin; NPC, nuclear pore complex; RanBP, Ran-binding protein; RanGDP, GDP-bound Ran; RanGTP, GTP-bound Ran; RanGAP1, Ran GTPase activating protein 1; GAP, GTPase activating protein; RBH, RanBP1-homologous; TB, transport buffer; GST, glutathione S-transferase; PAGE, polyacrylamide gel electrophoresis; GMPPNP, guanylyl imidophosphate.
Nup358 (also termed RanBP2) is a 358-kDa nucleoporin located at the tips of the cytoplasmic fibrils of the NPC (41, 42). Its domains include a leucine-rich region, four RanBP1-homologous (RH) domains, a zinc finger region, scattered FG and FXFG repeats characteristic of a subset of nucleoporins, and a cyclophilin-homologous domain (41, 42). RanGTP binds to Nup358 in overlay and microtiter plate assays (25, 41, 42). Electron microscopy of rat liver nuclear envelope preparations shows that Ran loaded with GTP or its nonhydrolyzable analog GMPPNP interacts with the cytoplasmic side of the NPC (25, 41). These findings are consistent with an interaction between RanGTP and Nup358 at the NPC. In addition, Nup358 appears to form a trimeric complex with RanGTP and Kapβ1 in vitro (43). The binding properties of Nup358 are reminiscent of those of RanBP1, suggesting that Nup358 may be involved in GTP hydrolysis at the NPC. On the other hand, the location of Nup358 at the tips of the cytoplasmic fibrils of the NPC suggests that it may be involved in the initial docking of substrate prior to import.

Many of the recent advances in characterizing nuclear import factors were accomplished by using fluorescent import substrates in a digitonin-permeabilized cell assay (44). One of the major tasks that currently face the field is dissecting the events that occur at the NPC during import (3), a challenge that cannot be met at the resolution level of this assay. Ultrastructural studies of import to date (19, 45–52) have focused mainly on microinjection experiments in intact cells, in which identifying the functions of individual factors is difficult.

In this study, we bring ultrastructural analysis and Ran GTPase activity measurements to the digitonin-permeabilized cell system. This approach is combined with in vitro binding and GTPase assays, as well as standard import assays, to examine where and how the Kap and GTPase activities, as well as standard import assays, to

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**EXPERIMENTAL PROCEDURES**

**Cell Culture and Permeabilization**—HeLa cells were cultured in Dulbecco’s modified Eagle’s medium supplemented by 10% fetal calf serum, 2 mM glucose, and 100 units/ml each of penicillin and streptomycin. For electron microscopy and GTPase assays, 50–80% confluent cells were digitonin-permeabilized and stored essentially as described (53). Cells were nonenzymatically dissociated in dissociation solution (Sigma C-5914) for 15 min at 37 °C and washed three times by low speed centrifugation in cold transport buffer (TB) (20 μM HEPES, pH 7.4, 110 mM potassium acetate, 2 mM magnesium acetate, 1 mM EGTA). They were then suspended in TB with 35 μM digitonin and incubated for 5 min on ice followed by three washes in cold TB. They were resuspended in TB with 10 mM Mg bovine serum albumin (Sigma A-7030) and 5% Me₂SO at 107 cells/ml and stored in aliquots at −80 °C.

**Recombinant Proteins**—Human Ran was expressed in *E. coli BL21(DE3)* from a pET-9C construct (29) and purified as described (21). Recombinant human Ran was loaded with either GDP, GTP, or GMPPNP as described (21) prior to use for gold conjugation, binding assays, or import assays. The murine RanGAP1 open reading frame was amplified by polymerase chain reaction from a construct kindly provided by Mark Rush (54) and subcloned into pGEX 4T3 (Amersham Pharmacia Biotech), from which it was expressed in *E. coli*. Recombinant human Nup358-1 was expressed in *E. coli* as a fusion protein, puriﬁed by glutathione-Sepharose, and eluted by thrombin cleavage according to the manufacturer’s instructions. The Kapβ1 open reading frame was ampliﬁed from a human cDNA library (CLONTECH), subcloned into pGEX4T3, expressed in *E. coli* as a GST fusion protein, and puriﬁed by binding to glutathione-Sepharose.

**GTPase Hydrolysis and Nucleoporin Nup358**

For electron microscopic and binding studies, Kapβ1 was eluted with 40 μM RanBP1 and 40 μM GTPase assay buffer, was eluted with glutathione-Sepharose, and eluted by thrombin cleavage according to the manufacturer’s instructions as a GST fusion protein. Murine Kapβ2 (55), expressed with a C-terminal His tag from a pET21A construct, was a kind gift from Michael Matunis. The Nup358-4 protein (12) was a kind gift from Jian Wu. The Nup358-1 construct was produced by amplifying the region of the Nup358 cDNA that encodes amino acids 996–1963 from a plasmid containing the 7-4 fragment of Nup358 (41) provided by Jian Wu. It was subeluted into pGEX4T3 and expressed as a GST fusion protein in *E. coli*. Recombinant RanBP1, expressed from a pET11 construct (kindly provided by Elias Coutavas), was a gift from Yuh-Min Choak and Tsong-Hark Park.

**Conjugation of Recombinant Proteins to Colloidal Gold Particles**—The optimum amount of recombinant protein needed to stabilize colloidal gold particles was determined by electrophoresis. According to the manufacturer’s instructions, colloidal gold beads, 10 nm in diameter, were incubated with the recombinant protein for 5 min at room temperature and brought up to pH 9 with 0.2 M potassium carbonate. Bovine serum albumin was added to 10 mg/ml and the coated beads were then centrifuged 50-fold by centrifugation according to the manufacturer’s instructions and stored at 4 °C for up to 1 month. Gold conjugates were checked by electron microscopy to ensure that the preparation is monodisperse with no clumping of gold particles.

**Electron Microscopy**—Approximately 5 × 104 digitonin-permeabilized HeLa cells were incubated with 1 μl of 10-nm gold conjugate for 40 min at 22 °C in 30 μl of TB followed by overnight fixation in 5% glutaraldehyde at 4 °C. Samples were processed for transmission electron microscopy and viewed with a Jeol 100CX microscope (Jeol USA, Peabody, MA). Two photographs per cell were taken from ultrathin sections at × 26,000 magnification from 5–6 cells for each experimental point. For quantitation of Kapβ1 binding to the NPC, the gold beads associated with all the morphologically preserved NPCs visible in the photograph (approximately 40 NPCs per sample) were counted and divided by the number of NPCs. All electron micrographs were scanned and analyzed using Adobe Photoshop 4.0 software. Gold particle analysis of the images was carried out with NIH Image 1.61 software.

**In Vitro Binding Assays**—Binding reactions contained 10 μl of glutathione-Sepharose beads (Amersham Pharmacia Biotech) in a total volume of 46 μl in transport buffer with Tween 20 (20 mM HEPES, pH 7.4, 110 mM potassium acetate, 2 mM magnesium chloride, and 0.1% Tween 20) and 0.5 mg/ml Pefabloc SC (Roche Molecular Biochemicals). Nup358-1 (1 μg/ml) was present in each reaction. Ran was loaded with [γ-32P]RanGTP as described (21) and used at 0.4 μM in all reactions. When Kapβ1 was used, it was preincubated at 0.25 μM for 30 min at 4 °C with the [γ-32P]RanGTP before the other components were added, in both in vitro and permeabilized cell assays. In vitro binding assay were done according to the following as indicated in Fig. 5A: 0.25 μM GST-Nup358-1, 0.6 μM Kapβ1, 1.2 μM NLS peptide (56), and 20 nM RanGAP1. For permeabilized cell assays using digitonin-permeabilized cells, 5 μl of cell extract were used per assay. Ten μM RanGAP1 in the presence or absence of 0.6 μM Kapβ1 and 1.2 μM NLS peptide, as indicated in Fig. 5B. All reactions were carried out in 45 μl of hydrolysis buffer (20 mM HEPES, pH 7.4, 100 mM...
slips for 24 h, permeabilized with 30 pM preequilibrated MicroSpin columns (Amersham Pharmacia Biotech). The filter was washed with 5 ml of cold hydrolysis buffer, and the buffer; half of the reaction was then passed through a nitrocellulose filter. The filter was washed with 5 ml of cold hydrolysis buffer, and the radioactivity retained on it was counted in a scintillation counter.

Import Assays—GST-Kap1 fusion protein was immobilized on glutathione-Sepharose beads, and an excess of either RanGTP or RanGMPPNP was added. After a 1-h incubation at 4 °C, the beads were washed twice, and the Kap1-RanGTP and Kap1-RanGMPPNP complexes were eluted with glutathione according to the manufacturer’s instructions. The buffer was changed to TB by centrifugation through preequilibrated MicroSpin columns (Amersham Pharmacia Biotech) prior to use in import reactions. HeLa cells were grown on glass coverslips for 24 h, permeabilized with 30 μg/ml digitonin in TB for 5 min at room temperature, and washed three times with transport buffer. Import reactions included 0.1 μM Kap1-RanGTP or Kap1-RanGMPPNP complexes, 0.6 mM Kap1, 5 mM GTP or GMPPNP in TB. The mixtures, except for RanGAP1, were preincubated for 15 min on ice. RanGAP1 was added immediately before the mixtures were added to permeabilized cells. Import was allowed to proceed for 15 min at room temperature, and cells were washed twice in TB, fixed in 2% formaldehyde in TB, and visualized using a Zeiss Axiohot microscope using a ×63 objective. Images were captured with Adobe Photoshop 4.0 software.

RESULTS

Karyopherin β1 Docks at the RBH Domains of Nup358 by Forming Trimeric Complexes with Either RanGDP or RanGTP—Karyopherin β1 (Kap1) binds to several nucleoporins in vitro (17, 57–62). Of these, the nucleoporin Nup358 is located at the tips of the cytoplasmic fibrils of the NPC, where the initial docking of import substrates is believed to occur prior to import (46, 49). It contains a leucine-rich region, four domains that are homologous to RanBP1 (RBH domains), eight zinc fingers, a cyclophilin-homologous domain, and scattered FG repeats that are characteristic of a subset of nucleoporins (41, 42) (Fig. 1). Trimeric complexes between RanBP1, Kap1, and either RanGTP or RanGDP have been demonstrated in solution binding assays (34, 35). This would suggest that trimeric complexes could be formed between Kap1, RBH domains, and either RanGDP or RanGTP. However, in overlay and microtiter plate assays, binding of Kap1 to Nup358 was found to be enhanced by RanGTP but not RanGDP (43).

Ran-dependent binding of Kap1 to Nup358 would be of potential significance for both import of NLS-bearing cargo and recycling of Kap1 after a round of import. RanGDP is required for import of Kapa and NLS-bearing cargo (19, 28) in addition to Kap1 (the so-called Kap1-Ran complexes involving Kap1, RanGDP, and Nup358) as intermediates in nuclear import. On the other hand, after a round of import, Kap1 is bound to RanGTP and probably exported as a Kap1-RanGTP complex. This complex may bind to Nup358 as its last station on the NPC before recycling. With these considerations in mind, we carried out solution binding assays to determine whether Kap1 can bind to Nup358 in the presence of RanGDP or RanGTP, and which domains of Nup358 are involved in such binding.

For this purpose, two recombinant fragments of Nup358 were expressed in E. coli as fusion proteins with an N-terminal GST tag (Fig. 1). The first fragment, called Nup358-1, contains the RBH1 domain, the eight zinc fingers, and several FG repeats. The second fragment, called Nup358-4, contains the RBH4 domain, the cyclophilin-homologous domain, and several FG repeats (Fig. 1). The constructs were intended to include as much representative sequence from Nup358 as possible within the constraints imposed by the bacterial expression system. We wanted to express the RBH domains in their natural context within Nup358 sequences and to include most of the FG repeats, which have been implicated in the binding of Kap1 to other nucleoporins (17, 57, 58, 60, 63, 64).

We first incubated immobilized Nup358-1 and Nup358-4 with Kap1 and either RanGDP or RanGTP. Bound and unbound material was analyzed by SDS-PAGE (Fig. 2). Very little Kap1 bound to either of the Nup358 fragments, despite the presence of FG repeats in both fragments; close inspection of the original gels reveals a faint band (see Fig. 7A, lane 2). However, in the presence of either RanGTP or RanGDP, there was strong binding of Kap1 to both Nup358-1 and Nup358-4 (Fig. 2). These data indicate that each of the two Nup358 fragments is capable of forming trimeric complexes with Kap1 and either RanGTP or RanGDP. When incubations were carried out in the presence of increasing concentrations of RanBP1, there was complete inhibition of Ran-mediated binding of karyopherin β1 to Nup358-1 and Nup358-4, indicating that karyopherin β1 and Ran form trimeric complexes with the RBH domains of Nup358 (Fig. 3).

The Preformed Kap1-RanGTP-RBH Complex Resists Dissociation by RanBP1—RanBP1 has been implicated in the recycling of Kap1-RanGTP complexes (36, 37). However, these complexes may encounter and bind to Nup358 on their way out of the NPC before they encounter RanBP1, because the latter is a cytosolic protein (65). It was therefore important to determine whether RanBP1 would be able to dissociate a Kap1-Ran-RBH complex after it is formed. Kap1 and either RanGDP or RanGTP were incubated with Nup358-4 immobilized on beads in order to form Kap1-RanGDP-RBH and Kap1-RanGTP-RBH complexes, respectively, as before. The beads were then washed and subjected to a second incubation with increasing concentrations of RanBP1 followed by SDS-PAGE of bound and unbound fractions (Fig. 4). The preformed Kap1-RanGTP-RBH trimeric complex was resistant to dissociation by RanBP1. In contrast, the preformed trimeric complex of Kap1-RanGDP-RBH was readily dissociated by RanBP1 (Fig. 4). Identical results were obtained with Nup358-1 (data not shown). Hence, once a trimeric RBH-Kap1-RanGTP complex is formed, RanBP1 is unable to disassemble the complex, whereas a trimeric complex of RBH-Kap1-RanGDP can be disassembled by RanBP1 (or presumably another RBH domain of Nup358). These results suggest that GTP hydrolysis is needed before
Kapβ1 can move from one RBH to the next or be released by RanBP1.

**Ultrastructural Analysis of Kapβ1 Docking at the NPC**—To assess the significance of these *in vitro* binding data in the context of intact NPCs, Kapβ1 was coupled to 10-nm colloidal gold and incubated with digitonin-permeabilized cells, either alone or in the presence of RanGDP or RanGTP. Electron microscopic inspection of sections of these cells confirmed the *in vitro* binding data (Fig. 5). There was very little binding to NPCs of Kapβ1-gold alone (Fig. 5, top panel), whereas significant gold decoration of NPCs occurred when either RanGDP (Fig. 5, middle panel) or RanGTP (Fig. 5, bottom panel) was present during the incubation. Localization of the gold particles to the cytoplasmic fibrils of the NPC and competition by RanBP1 (see below) indicate that they are indeed decorating Nup358.

Electron microscopic analysis also confirmed the *in vitro* competition data with concomitantly added RanBP1 (data not shown) and with RanBP1 added after docking (Fig. 6). Kapβ1-gold was first allowed to dock in digitonin-permeabilized cells, either alone or with RanGDP or RanGTP. This was followed by a second incubation in the presence or absence of RanBP1, and the average number of Kapβ1-gold particles per NPC under each condition was then determined. There was considerable reduction of NPC-bound gold particles when a preformed Kapβ1-gold-RanGDP-Nup358 complex was exposed to RanBP1, whereas the preformed Kapβ1-gold-RanGTP-Nup358 complex remained stable (Fig. 6). Interestingly, Kapβ1 binding that occurred in the absence of exogenously added Ran was also diminished by RanBP1 treatment, suggesting that this binding is mediated in part by the formation of a trimeric complex with residual endogenous RanGDP and Nup358. In fact, residual Ran can be seen at the nuclear envelope in digitonin-permeabilized cells by immunofluorescence (66).

Thus, the binding of Kapβ1-RanGTP to Nup358 is stable both *in vitro* and in permeabilized cells, whereas Kapβ1 docked through RanGDP can be readily displaced. These results suggest that once a Kapβ1-RanGTP complex resulting from one round of import binds to Nup358, another round of NLS import may not be possible before GTP hydrolysis occurs.

**Induction of GTP Hydrolysis by Kapα and NLS**—The Kapβ1-RanGTP complex is resistant to GTP hydrolysis by RanGAP (21). RanBP1 can form a trimeric complex with Kapβ1 and RanGTP *in vitro* (34, 35), and in this context, the addition of Kapa allows GTP hydrolysis in the presence of RanGAP (36, 26496 GTP Hydrolysis and Nucleoporin Nup358
GTP Hydrolysis and Nucleoporin Nup358

Ultrastructural studies (46, 49) have suggested that the initial docking site in nuclear import is at the tips of the cytoplasmic fibers of the NPC. Because this is where Nup358 is localized as well (41), the NLS-Kapb1-RanGTP-Nup358 complexes may represent initial docking events in classical NLS-mediated nuclear import.

To test whether GTP hydrolysis could be induced within the trimeric and higher order RanGTP-containing complexes described above, we preincubated Kapb1 and RanGDP or RanGTP at 4 °C to allow binary complex formation and then incubated the Kapb1-RanGTP complex at 22 °C with RanGAP1 and various combinations of Kapb, NLS peptide, and Nup358-1. The reactions were stopped and passed through a nitrocellulose filter, and the amount of unhydrolyzed Ran-bound GTP was measured by counting radioactivity that remained bound to the filter after washing (21). The Kapb1-RanGTP-Kapb1 trimeric complexes were resistant to RanGAP1 activity (Fig. 8A).

Addition of Kapb resulted in partial GTP hydrolysis, and when NLS peptide was included in the reaction, complete hydrolysis occurred (Fig. 8A). This is consistent with the cooperative binding of Kapb and NLS peptide in the formation of pentameric complexes (see above). The results were identical when Kapb with or without NLS peptide was added to a preformed Kapb1-RanGTP-Nup358 complex (data not shown). In addition, identical results were obtained when RanBP1 was substituted for Nup358-1 (data not shown). Thus, the presence of transport cargo favors the hydrolysis of GTP, consistent with a role for GTP hydrolysis by Ran during nuclear import.

To study GTP hydrolysis by Ran within intact NPCs in a context closer to the in vivo situation, we used digitin-permeabilized HeLa cells (Fig. 8B). RanGAP1 exists in cells in two forms, a free cytosolic form and an NPC-bound form that is covalently modified with a small ubiquitin-like peptide (SUMO-1) (10, 11). The latter form (SUMO-RanGAP1) is attached to the C terminus of Nup358 and is the only species retained in digitin-permeabilized cells (10, 13). Assays were carried out in a manner similar to the in vitro assays with preincubation of [γ-32P]GTP with or without Kapb1. GTP hydrolysis assays were then started by adding digitin-permeabilized cells with or without Kapb and NLS peptide (Fig. 8B). The digitin-permeabilized cells served as a source of both Nup358 and NPC-associated SUMO-RanGAP1. When RanGTP was added to digitin-permeabilized cells in the absence of Kapb1, GTP was readily hydrolyzed (Fig. 8B). When RanGTP was preincubated with Kapb1, there was maximal inhibition of GAP activity. Thus SUMO modification and NPC association of RanGAP1 do not override the inhibitory effect of Kapb1 on GTP hydrolysis. As in the in vitro experiment, addition of Kapb stimulated partial GTP hydrolysis. The addition of NLS did not further stimulate GTP hydrolysis by NPC-associated SUMO-RanGAP1 (Fig. 8B).

The presence of residual unhydrolyzed GTP under these conditions suggests that some Kapb1-RanGTP associated with the four RBH domains of Nup358 might not be accessible to SUMO-RanGAP1, which is attached to the C terminus of Nup358. Indeed, this residual GTP was completely hydrolyzed in a Kapb and NLS-dependent fashion when recombinant free RanGAP1 was included in addition to the permeabilized cells (data not shown). Thus, the two forms of RanGAP—soluble and NPC-associated—may play complementary roles in GTP hydrolysis at the NPC.

GTP Hydrolysis Is Required for Recycling of the Kapb1-RanGTP Complex for Further Rounds of Import—Several previous studies have shown that import of classical NLS bearing substrates requires GTP hydrolysis by Ran (22–28). On the other hand, a recent study indicated that GTP hydrolysis might not be required for the translocation step of the nuclear import...
of classical NLS-bearing proteins (38). In that study, Ran was supplied in its GDP-bound form in the import reactions. However, as discussed previously, Ran is likely to be in the GTP bound form and in complex with Kapβ1 at the end of a round of import; and GTP hydrolysis may then be required for a recycling step before this Kapβ1-RanGTP complex can be utilized for another round of import. To address this question, RanGTP or Ran loaded with the nonhydrolyzable GTP analog GMPPNP was added in excess to GST-Kapβ1 immobilized on glutathione-Sepharose beads. After washing to remove unbound Ran, the Kapβ1-RanGTP and Kapβ1-RanGMPPNP complexes thus formed were eluted from the beads with glutathione (Fig. 9A) and used in standard import reactions in permeabilized cells in the presence of free GTP and GMPPNP, respectively (Fig. 9B). As shown in Fig. 9B, the Kapβ1-RanGTP complex supported import, whereas the Kapβ1-RanGMPPNP complex did not. These data show that GTP hydrolysis is required for recycling of a Kapβ1-RanGTP complex and reinitiation of nuclear import of a classical NLS-bearing substrate.

DISCUSSION

Nuclear import of classical NLS-bearing proteins is mediated by a Kapα-β1 heterodimer. Other soluble factors involved in the process include Ran, p10, RanBP1, RanGAP1, and GTP. Binding of RanGTP to Kapβ1 dissociates Kapα from Kapβ1, resulting in the release of Kapα and its NLS-bearing cargo into the

FIG. 7. Kapα and NLS can form a pentameric complex with Nup358, Kapβ1, and either RanGDP or RanGTP. A, Kapα and NLS bind to Nup358 in a Kapβ1- and Ran-dependent fashion. Glutathione-Sepharose beads with immobilized Nup358-4 at 0.25 μM were incubated with 0.25 μM Kapβ1, 0.6 μM Kapα, 1.2 μM NLS peptide, and 0.8 μM RanGDP or RanGTP as indicated. Bound fractions were visualized by SDS-PAGE, Western blotting, and Amido Black staining. Kapα was also visualized by Ni-NTA horseradish peroxidase treatment followed by chemiluminescence detection (see under “Experimental Procedures”). Unbound fractions were visualized by SDS-PAGE and Coomassie Blue staining. B, Kapα and NLS bind cooperatively in the formation of pentameric NLS-Kapα-Kapβ1-RanGTP-Nup358 complexes. Glutathione-Sepharose beads with immobilized Nup358-4 at 0.25 μM were incubated with 0.25 μM Kapβ1 and 0.6 μM Kapα in the presence or absence of 1.2 μM NLS peptide, 0.8 μM RanGDP or RanGTP as indicated. Bound Kapα was visualized by Ni-NTA horseradish peroxidase treatment followed by chemiluminescence detection (see under “Experimental Procedures”). C, the blot shown in B was subjected to densitometric analysis with Scan Analysis version 2.56 software. The amount of Kapα is expressed in arbitrary units.
nucleus. In this study, we analyze how and where the Kapβ1-RanGTP complex is recycled at the NPC to initiate another round of import. Data from in vitro binding and GTPase activity assays are correlated with findings from ultrastructural and functional assays in permeabilized cells to provide a clearer picture of the events that actually occur on intact NPCs. With this approach, we show that the RBH domains are a site where docking of NLS cargo is linked to GTP hydrolysis by Ran and that this hydrolysis is a prerequisite for repeated cycles of import.

Ran-dependent Docking of Kapβ1 on RBH Domains at the NPC—Kapβ1 is involved in the docking of NLS-bearing proteins at the NPC. In vitro and in cell lysates, Kapβ1 has been shown to bind to several nucleoporins (17, 34, 36, 43, 57–62, 64). In several cases, binding of Kapβ1 has been localized to the FG repeat regions of nucleoporins (17, 57, 58, 60, 63, 64), and in another case, it has been localized to the α-helical coiled-coil rod domain of the nucleoporin p62 (61).

After translocation through the NPC, RanGTP binds to Kapβ1, leading to the release of the Kapβ1-NLS complex in the nucleus. The mechanism of reexport of Kapβ1 after a round of nuclear import is not well understood. A leucine-rich nuclear export sequence in yeast Kapβ1 has been reported to mediate its export to the cytoplasm and its interaction with GLFG-containing yeast nucleoporins (63). It is not known whether other soluble export factors are involved in Kapβ1 export, but it is probably reexported in a complex with RanGTP (20). The Kapβ1-RanGTP complex is then presumably recycled to initiate further rounds of import. In vitro, binding of RanGTP to Kapβ1 releases it from some nucleoporins (17, 43, 61), but it enhances its binding to Nup358 (Fig. 2) (43). Nup358 is the outermost known nucleoporin on the cytoplasmic side of the NPC and is likely to be the first station for nuclear import of NLS-bearing proteins as well as the last station for the reexported Kapβ1-RanGTP complex on the NPC.

Our data (Figs. 4 and 5) show that either RanGDP or RanGTP can target Kapβ1 to Nup358 by forming trimeric complexes and that the bulk of Kapβ1 docking on Nup358 is Ran-dependent. Furthermore, although RanGDP can bind either to the zinc fingers or the RBH domains of Nup358 (67), Ran-dependent docking of Kapβ1 occurs through the RBH sites only, as evidenced by the fact that this binding can be prevented by concomitantly added excess RanBP1 (Fig. 3). Ultrastructural studies with Kapβ1-gold conjugates in digitonin-permeabilized cells confirm RanGDP- and RanGTP-dependent docking of Kapβ1 at the cytoplasmic fibrils (Fig. 5) and competition by RanBP1 (Fig. 6). It should be noted that, although RanBP1 inhibits Kapβ1 binding to Nup358, it enhances docking and import of NLS-bearing proteins (34). The mechanism of this enhancement is not clear; it is possible that RanBP1 stabilizes NLS docking at NPC sites other than the RBH domains of Nup358.
that once it is formed, it resists dissociation by subsequently added RanBP1 both \textit{in vitro} and on intact NPCs, indicating that it is relatively stable (Figs. 4 and 6). The RanGDP-containing complex, on the other hand, is readily dissociated by subsequently added RanBP1. These data imply that GTP hydrolysis by Ran is needed in order to allow movement of Kap$\beta_1$.

Docking of KapoNLS, GTP Hydrolysis, and Import—The stability of the Kap$\beta_1$-RanGTP-Nup358 complex in comparison to the Kap$\beta_1$-RanGDP-Nup358 complex (see above) suggested that GTP hydrolysis is required before translocation and import can occur. Indeed, nonhydrolyzable analogs of GTP and mutants of Ran that do not hydrolyze GTP have been reported to inhibit nuclear import (22–28). On the other hand, a recent study showed that the nonhydrolyzable GTP analog GMPPNP can support classical NLS-mediated import in the presence of RanGDP (38). However, a round of nuclear import results in a Kap$\beta_1$-RanGTP complex, and it is this complex that needs to be recycled and used in a subsequent round of import. A hydrolysis requirement for recycling would be bypassed if RanGDP were used as a starting point. Indeed, as shown in Fig. 9, when cells were presented with a Kap$\beta_1$-RanGMPPNP complex, import failed to occur, whereas the Kap$\beta_1$-RanGTP complex was able to support import under similar conditions.

Our studies of GTP hydrolysis \textit{in vitro} and in permeabilized cells (Fig. 8) revealed a requirement for Kapo and NLS peptide for maximal GTP hydrolysis at the NPC. The trimeric Kap$\beta_1$-RanGTP-Nup358 complex was resistant to the action of RanGAP1. The addition of Kapo and NLS peptide cooperatively induced complete GTP hydrolysis in the presence of RanGAP1. This did not result from a dissociation of Kap$\beta_1$ from Nup358 and RanGTP, but rather from cooperative binding of Kapo and NLS to the Kap$\beta_1$-RanGTP-Nup358 complex (Fig. 7). Ultrastructural studies indicate that the initial docking site of NLS bearing cargo is at the tips of the cytoplasmic fibrils (46, 49), where Nup358 resides. Thus, the pentameric NLS-Kapo-Kap$\beta_1$-Ran-Nup358 complexes may represent early intermediates in the nuclear import pathway for classical NLS-bearing proteins (Fig. 10). The small amount of Kapo-NLS binding in the absence of Ran could be mediated by interaction of Kap$\beta_1$ with the repeat regions present in our Nup358 constructs (Fig. 1), but the significant increase seen in the presence of Ran, particularly RanGTP, occurs in all probability through the RBH domains. However, even in the presence of RanGTP, the amount of Kapo-NLS bound was small compared with the input, suggesting that the pentameric complexes represent unstable transient intermediates in nuclear import, the function of which is to induce GTP hydrolysis. In summary, the RBH domains of Nup358 appear to be a site where docking of Nup358 complex was resistant to the action of RanGAP1. However, when they added recombinant Kap$\beta_1$ to isolated nuclear envelopes and detected it by immunoelectron microscopy, the substantial amount of Kap$\beta_1$ docking on the cytoplasmic side of the NPC was reduced by RanGTP instead of the increase one might have expected. This is probably due to the presence of other nucleoporins on the cytoplasmic side of the NPC and the fact that RanGTP dissociates Kap$\beta_1$ from some nucleoporins (see above). Our data, on the other hand, were obtained by directly coupling recombinant Kap$\beta_1$ to colloidal gold and applying it to digitonin-permeabilized cells with or without Ran (Fig. 5). We saw little docking in the absence of Ran, but docking was markedly enhanced in the presence of either RanGDP or RanGTP (Figs. 5 and 6). It thus appears that gold conjugation of Kap$\beta_1$ has led to a reduction in docking on other nucleoporins while leaving Ran-dependent docking on RBH domains relatively intact. This may be due to selective masking of certain domains on Kap$\beta_1$ or to steric interference by the bulk of the gold particle. In any case, this has allowed us to examine docking on Nup358 in relative isolation from the background of other nucleoporins. We have thus been able to confirm the \textit{in vitro} binding and competition data and to show that Ran-dependent docking of Kap$\beta_1$ occurs on intact NPCs.

As shown diagrammatically in Fig. 10, formation of a Kap$\beta_1$-RanGTP-Nup358 complex is likely to be the last step in the reexport of the Kap$\beta_1$-RanGTP complex through the NPC. An important feature of the Kap$\beta_1$-RanGTP-Nup358 complex is

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**Fig. 9.** GTP hydrolysis is required for reinitiation of nuclear import. A. preparation of Kap$\beta_1$-RanGTP and Kap$\beta_1$-RanGMPPNP complexes. RanGTP or RanGMPPNP was added in excess to GST-Kap$\beta_1$ immobilized on glutathione-Sepharose beads. After washing, the Kap$\beta_1$-RanGTP and Kap$\beta_1$-RanGMPPNP complexes were eluted with glutathione, exchanged into transport buffer, and checked by SDS-PAGE and Coomassie Blue staining. B. recycling of the Kap$\beta_1$-RanGTP complex for nuclear import requires GTP hydrolysis. Import reactions were carried out in permeabilized cells as described under “Experimental Procedures.” In the left panel, Kap$\beta_1$-RanGTP complex and GTP were added, whereas the right panel contained Kap$\beta_1$-RanGMPPNP complex and GMPPNP. In addition, both reactions contained NLS-bearing bovine serum albumin-rhodamine, Kapo, p10, RanBP1, and RanGAP1.

It is of interest in this context to compare our data to those of Delphin et al. (43). Their overlay and microtiter plate assays (in agreement with our solution binding assays) show that RanGTP enhances the binding of Kapo to Nup358. However, when they added recombinant Kap$\beta_1$ to isolated nuclear envelopes and detected it by immunoelectron microscopy, the substantial amount of Kap$\beta_1$ docking on the cytoplasmic side of the NPC was reduced by RanGTP instead of the increase one might have expected. This is probably due to the presence of other nucleoporins on the cytoplasmic side of the NPC and the fact that RanGTP dissociates Kap$\beta_1$ from some nucleoporins (see above). Our data, on the other hand, were obtained by directly coupling recombinant Kap$\beta_1$ to colloidal gold and applying it to digitonin-permeabilized cells with or without Ran (Fig. 5). We saw little docking in the absence of Ran, but docking was markedly enhanced in the presence of either RanGDP or RanGTP (Figs. 5 and 6). It thus appears that gold conjugation of Kap$\beta_1$ has led to a reduction in docking on other nucleoporins while leaving Ran-dependent docking on RBH domains relatively intact. This may be due to selective masking of certain domains on Kap$\beta_1$ or to steric interference by the bulk of the gold particle. In any case, this has allowed us to examine docking on Nup358 in relative isolation from the background of other nucleoporins. We have thus been able to confirm the \textit{in vitro} binding and competition data and to show that Ran-dependent docking of Kap$\beta_1$ occurs on intact NPCs.

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provided in its GTP-bound form (19). Furthermore, yeast RanGAP (Rna1p), which is neither SUMO-modified nor targeted to the NPC, has been shown to be essential for import (68).

Another difference between NPC-associated SUMO-RanGAP1 and free unmodified RanGAP1 is that NLS peptide did not enhance GTP hydrolysis by the former (Fig. 8B). A comparison with yeast may be relevant to this observation. Hydrolysis of GTP after the formation of a yeast Kapβ1-RanGTP complex can be accomplished in a reaction involving RanBP1, Kapα, and the C terminus of the yeast nucleoporin Nup1 (36). It is interesting to note that in the same study, direct binding of the Kapα NLS to RanGAP1 remains to be determined. One possibility is that hydrolysis occurs within a pentameric NLS-Kapβ1 complex forms in the cytosol and then docks to the Kapα and NLS release Kapβ1 from RanGTP, and that this is followed by the formation of a more stable RanGDP-containing complex (70). Our data agree with the second model in that GTP hydrolysis occurs on Nup358. However, we show that Kapα and NLS bind to the Kapβ1-RanGTP-Nup358 complex rather than destabilize it, that RanGTP is more favorable for this binding than RanGDP, and that hydrolysis occurs within a pentameric NLS-Kapα-Kapβ1-RanGTP-Nup358 complex.

To summarize, we have shown that Nup358 at the cytoplasmic fibers of the NPC provides a site where GTP hydrolysis by Ran terminates one cycle of import as Kapα and NLS dock to initiate the next cycle. This may be the first and last of several steps at the NPC that are required to complete a cycle of nuclear import.

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