The Nucleoporin Nup98 Is a Site for GDP/GTP Exchange on Ran and Termination of Karyopherin β2-mediated Nuclear Import*

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Karyopherin β2 (Kap β2, transportin) binds the M9 sequence of human ribonucleoprotein A1 and mediates its nuclear import. Here we show a role for the nucleoporin Nup98 in the disassembly of Kap β2 import complexes at the nuclear side of the nuclear pore complex (NPC). Kap β2 bound to a region at the N terminus of Nup98 that contains an M9-like sequence. The human ribonucleoprotein A1 M9 sequence competed with Nup98 for binding to Kap β2, indicating that Nup98 can dissociate Kap β2 from its substrate. Binding of Kap β2 to Nup98 was inhibited by Ran loaded with guanylyl imidophosphate, suggesting that RanGTP dissociates Kap β2 from Nup98. RanGTP is produced from RanGDP through nucleotide exchange mediated by RanGEF (RCC1). Immunoelectron microscopy and nucleotide exchange assays revealed functional RanGEF on both sides of the NPC. On the nuclear side, the localization of RanGEF coincided with that of Nup98. RanGEF bound to Nup98 at a region adjacent to the Kap β2-binding site. These findings suggest a model where 1) import substrate is released from Kap β2 at the nucleoplasmic side of the NPC by competition with the Nup98 M9-like site, 2) Nup98-bound RanGEF catalyzes the formation of RanGTP, and 3) RanGTP dissociates Kap β2 from Nup98 allowing repeated cycles of import.

Transport of proteins and nucleic acids between the nucleus and cytoplasm occurs through nuclear pore complexes (NPCs)1 (1). The vertebrate NPC is a supramolecular structure of 125 million daltons, which is approximately 30 times the size of a ribosome (2). Each NPC is composed of a central cylinder surrounded by a spoke-ring structure that anchors the cylinder to the nuclear envelope (3, 4). Filaments extending 50–100 nm emanate from this central core into the cytoplasm and nucleoplasm (5, 6). On the nucleoplasmic side, these filaments form a basket-like structure (7). In the mammalian NPC, all these structures together consist of approximately 50 different proteins termed nucleoporins (8).

A subset of nucleoporins that contain Pehe-Gly repeats (FG nucleoporins) constitute docking sites for transport factors at the NPC (9–12). Some of these nucleoporins are localized asymmetrically at the NPC. Nup358 and Nup214, for example, are associated with the cytoplasmic filaments, whereas Nup98 and Nup153 are localized at or near the basket on the nucleoplasmic side (10, 13–16). On the other hand, p62 appears to be symmetrically localized on both sides close to the midplane of the NPC (17, 18). The asymmetric distribution of nucleoporins and their different affinities for import and export complexes may be important in determining the directionality of transport.

Nup98 contains a number of FG repeats in its N-terminal portion that act as docking sites during import (10). In addition, Nup98 appears to be involved in multiple RNA export pathways (19, 20). Interestingly, Nup98 has been described as a frequent target for chromosomal rearrangements in acute leukemia, and its N-terminal FG repeat region is present in all leukemia-associated Nup98 fusions that have been characterized to date (21–32). These findings suggest a link between the function of Nup98 in nuclear transport and its role in leukemogenesis.

Nuclear import and export of molecules involve interactions of soluble transport factors with their cargos and with the NPC. Transport receptors bind import or export signals present in their cargos and are collectively known as karyopherins (also called importins or exportins). Nuclear import of proteins bearing a classical nuclear localization signal (cNLS) was the first to be characterized at the molecular level. Import of a cNLS-bearing protein is mediated by a heterodimer of karyopherin α (Kap α) and karyopherin β1 (Kap β1). Kap α interacts directly with the cNLS, whereas Kap β1 binds to nucleoporins resulting in the docking of the complex to the NPC. Other soluble factors are involved in the translocation process through the NPC, including the small GTPase Ran, p10 (also known as NTF2), and the Ran-binding protein RanBP1 (33–35).

Other members of the Kap β family serve as import receptors for other classes of proteins that have non-classical NLSs. In many of these cases the Kap β binds directly to the NLS of its import cargo rather than through a Kap-α-like adapter. For example, hnRNP A1 has an NLS rich in aromatic residues and glycine, called M9, that is directly bound by Kap β2 (transportin)1 resulting in docking of the complex at the NPC and its import into the nucleus (36, 37). Additional import and export pathways have been identified and are reviewed elsewhere (33–35).

Ran is a small GTPase that cycles between a GDP-bound form (RanGDP) and a GTP-bound form (RanGTP) and plays an

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1 The abbreviations used are: NPC, nuclear pore complex; NLS, nuclear localization signal; cNLS, classical nuclear localization signal; Kap, karyopherin; RanBP, Ran-binding protein; RanGDP, GDP-bound Ran; RanGTP, GTP-bound Ran; GMPPNP, guanylyl imidophosphate; RanGAP, Ran guanine nucleotide exchange factor; RCC1, regulator of chromosome condensation I; RanGAP1, Ran GTPase activating protein I; FG repeats, Phe-Gly repeats; GST, glutathione S-transferase; hnRNP, human ribonucleoprotein; PC, polymerase chain reaction; MBP, maltose-binding protein; DTT, dithiothreitol; PAGE, polyacrylamide gel electrophoresis; BSA, bovine serum albumin.
important role in both import and export (38–40). Interconversion of RanGDP and RanGTP is regulated by the Ran GTPase-activating protein RanGAP1 and the Ran guanine nucleotide exchange factor RanGef (also called RCC1). RanGAP1 is localized in the cytosol and at the cytoplasmic face of the NPC and catalyzes nucleotide hydrolysis by RanGTP to form RanGDP (41). RanGef is localized predominantly in the nucleus and catalyzes nucleotide exchange favoring the generation of RanGTP, since the intracellular concentration of GTP is higher than that of GDP (39, 40, 42–44). These findings suggest that the nucleus has a higher concentration of RanGTP than the cytoplasm. Ran appears to be imported primarily in the GDP-bound form, but functional RanGef is required in order for Ran to accumulate in the nucleus, presumably as RanGTP (45).

The mechanisms by which import complexes travel through the NPC and are disassembled in the nucleus are not well understood. The process is thought to involve several steps. Kapβs interact strongly with RanGTP but not with RanGDP. Binding of RanGTP to import Kapβs results in the release of their cargo. Since a higher concentration of RanGTP is predicted in the nucleus, it is thought that this reaction results in the release of cargo from import Kapβs in the nucleus (46–48). However, there is evidence that Kapβ2 can deliver its cargo to the nucleus in the absence of Ran when Kapβ2 and the cargo are provided in equimolar concentrations (49). Thus the release of cargo from the nuclear side of the nuclear pore complex may also occur by Ran-independent mechanisms.

In this study, we have focused on the role of the nucleoporin Nup98 in the termination of Kapβ2-mediated nuclear import at the nuclear side of the NPC. We show that Kapβ2 binds to the N terminus of Nup98 at a region that contains an hnRNP A1 M9-like sequence. This binding is prevented by an M9-containing protein, indicating that Nup98 binds to the cargo-binding site of Kapβ2. Thus, Nup98 may disassemble the Kapβ2-M9 import complex at the nuclear side of the NPC providing a mechanism for the Ran-independent import by Kapβ2 mentioned above. The resulting Kapβ2-Nup98 complex would need to be disassembled before Kapβ2 can be recycled for further rounds of import. This function is probably performed by RanGTP since our data show that RanGMAPPNP inhibits binding of Kapβ2 to Nup98. However, since Ran traverses the NPC on its way to the nucleus primarily in the GDP-bound form (45, 50), a mechanism is needed for the local production of RanGTP in order to dissociate Kapβ2 from Nup98. RanGef is the only known nucleotide exchange factor for Ran and is thought to be confined to the inside of the nucleus in association with chromatin (51). Here we show that RanGef, in addition to its intranuclear localization, is also associated with the NPC. By using immunoelectron microscopy, we show that RanGef is present on both the cytoplasmic and nucleoplasmic sides of the NPC. On the nucleoplasmic side, the localization of RanGef coincides with the previously determined localization of Nup98 (10). By using in vitro nucleotide exchange assays we found that nuclear envelope-associated RanGef is functional. Consistent with the ultrastructural data, RanGef indeed bound to Nup98, at the FG repeat region immediately downstream of the Kapβ2-binding site. These data are integrated into a model for the termination of Kapβ2-mediated nuclear import at the nuclear side of the NPC.

**EXPERIMENTAL PROCEDURES**

**Plasmid Construction and Protein Expression**—Wild-type Nup98 precursor was cloned into pα-MAX as described previously (52). All truncated mutants were generated by PCR using the wild-type Nup98 precursor as template. An oligonucleotide complementary to the 5' end of the indicated region for each mutant (see Fig. 3) and containing a SacI site was used in conjunction with an antisense oligonucleotide complementary to sequences at the 3' end of the indicated regions in Fig. 3 and containing a NotI site. The resulting PCR products were digested with SacI and NotI and ligated into the SacI/NotI sites of myc-α-MAX. The human Nup98 open reading frame was amplified by PCR from a bone marrow cDNA library (CLONTECH, Palo Alto, CA) and subcloned into the HindIII site of pGEX-4T3 (Amersham Pharmacia Biotech) to produce a GST-Nup98 fusion. The GST-Nup98 fusion protein was purified from bacterial lysate by binding to glutathione-Sepharose beads (Amersham Pharmacia Biotech).

The human Kapβ2 gene subcloned into pGEX4T3 containing a Tev protease cleavage site, and the maltose-binding protein fused with M3 MBP-Ntrk2 (53). Expression and purification of GST-Kapβ2 was performed as originally described (53). The maltose-binding protein (MBP) was obtained from New England Biolabs (Beverly, MA). The human RANGef open reading frame was amplified by PCR from a bone marrow cDNA library (CLONTECH, Palo Alto, CA) and subcloned into the BamHI and SacI sites of pGEX4T3 to produce a GST-RanGef fusion protein. The GST-RanGef fusion protein was purified from bacterial lysate by binding to glutathione-Sepharose beads and either kept immobilized on the beads or eluted by thrombin cleavage. Human recombinant Ran protein loaded with either GDP or GMPPNP was a kind gift from Yuh-Min Chook (53).

**In Vitro Binding Assays**—All wild-type and Nup98 mutant proteins were in vitro transcribed and translated using a coupled reticulocyte lysate transcription/translation system (Promega Corp., Madison, WI). Binding reactions were carried out as described (54) using 10 μl (unless otherwise indicated) in vitro transcribed and translated proteins as indicated in the figure legends. Bound and unbound fractions were separated on SDS-PAGE, and the gels were analyzed by autoradiography. Binding reactions with bacterially expressed recombinant proteins were carried out as described previously (54).

**Isolation and Fractionation of Nuclear Envelopes**—Rat liver nuclei were isolated as described (55) and stored frozen at –80°C in 100-unit aliquots (1 unit = 3 × 10⁶ nuclei). Nuclear envelopes were prepared by a modification of the procedure described by Dwyer and Blobel (56). Nuclei were thawed and pelleted at 500 rpm in a tabletop microcentrifuge for 1 min. After removing the supernatant, the pellet was resuspended to a final concentration of 100 units/ml by dropwise addition of cold buffer A, 0.1 mM MgCl₂, protease inhibitors (0.5 mM phenylmethanesulfonyl fluoride, 1 μg/ml leupeptin, 1 μg/ml pepstatin A, and 18 μg/ml aprotinin), 5 μg/ml DNase I (Sigma), and 5 μg/ml RNase A (Sigma) with constant vortexing. The nuclei were then immediately diluted to 20 units/ml by addition of ice-cold buffer B, 10% sucrose, 20 μM triethanolamine (pH 8.0), 50 μM MgCl₂, 1 mM dithiothreitol (DTT), and protease inhibitors, again with constant vortexing. Following a 15-min incubation on ice, the suspension was underlayered with 5 ml of ice-cold buffer C, 30% sucrose, 20 μM triethanolamine (pH 7.5), 0.1 mM MgCl₂, 1 mM DTT, and protease inhibitors, and centrifuged at 4,100 × g in a swinging bucket rotor (Sorvall type HB-4) for 15 min at 4°C. After removing the supernatant and sucrose cushion, the pellet was resuspended to a final concentration of 100 units/ml in ice cold buffer D, 10% sucrose, 20 μM triethanolamine (pH 7.5), 0.1 mM MgCl₂, 1 mM DTT, and protease inhibitors. The suspension was immediately underlayered with 5 ml of buffer C and pelleted as above. The pellet resulting from this second extraction is operationally defined as the nuclear envelope fraction.

**Immungold Electron Microscopy**—Isolated nuclear envelopes were fixed for 15 min in 2.5% formaldehyde in STM (10% sucrose, 20 μM triethanolamine HCl (pH 7.5), 0.1 mM MgCl₂) and centrifuged at 2,000 × g for 5 min onto 35-mm tissue culture dishes. The pelletted nuclear envelopes were washed three times with 1% BSA, 68 μM NaCl, 13 mM KCl, 15 mM KH₂PO₄, 40 mM NaHPO₄, 0.5 mM phenylmethane-sulfonil fluoride and incubated with two goat polyclonal anti-RanGef antibodies, RCC1(N-191) and RCC1(C-20), diluted 1:10 (Santa Cruz Bio-technology). Bound antibodies were detected with rabbit anti-goat IgG conjugated with 5-nm gold (Ted Pella, Redding, CA) diluted 1:50.

**Nucleotide Exchange Reactions**—Recombinant human Ran was prepared and loaded with [α-³²P]GTP as described (57). Exchange reactions were carried out at room temperature for 30 min in 300 μl of STM in the presence of 1 μM GDP. The reactions were stopped by adding 300 μl of ice-cold transport buffer with 1 mM DTT and 1 μg/ml BSA and applied onto BA85 filters (Schleicher & Schuell) by vacuum aspiration. The filters were washed with 5 ml of cold transport buffer, 1 mM DTT, 1 mg/ml BSA and allowed to dry before scintillation counting.

**RESULTS**

The N Terminal of Nup98 Contains an M9-like Sequence That Binds Karyopherin β2—We have previously shown that
Nup98 is synthesized as a precursor derived from at least two alternatively spliced mRNAs (52). The Nup98 precursor is cleaved at the C-terminus, generating Nup98 (residues 1–863) and a 6-kDa C-terminal fragment (residues 864–920), and the Nup98–Nup96 precursor is cleaved at the same site generating Nup98 (residues 1–863) and Nup96–(residues 864–1712) (52). Nup98 can be divided into two domains (Fig. 1A). The N-terminal half contains the FG-containing repeat region (residues 1–497), and the second half of the molecule contains the highly conserved cleavage site domain.

An alignment search showed that the N-terminal domain of Nup98 contains an hnRNP A1 M9-like sequence between residues 25–60 (Fig. 1). Using the Clustal method, the M9 sequence of hnRNP A1 and the M9-like sequence of Nup98 are approximately 30% identical and 56% similar (Fig. 1B). No other sequence similarity was observed between Nup98 and hnRNP A1. An M9-like sequence has previously been reported on Nup153 (58). This sequence has approximately 20% identity and 47% similarity to that of Nup98 M9 (Fig. 1A). The M9-like sequence in Nup98 is contained within a larger region that includes and extends beyond the M9-like region. The locations of the hnRNP A1 M9 and M3 sequences and the Nup98 M9-like sequence are indicated by thick lines. Boxes and shadings indicate sequence identity and homology, respectively.

Nup98 and Kapβ2 Compete for the Binding to Kapβ2—The presence of an M9-like sequence in Nup98 led us to investigate whether Nup98 would compete with M9 for binding to Kapβ2. The M9 sequence of hnRNP A1 is contained within a larger sequence, called M3 (Fig. 1A), that appears to bind to Kapβ2 more efficiently (37). The M3 fragment was expressed in bacteria as a fusion protein with MBP and used in the competition studies. In vitro binding assays were performed with immobilized GST–Kapβ2 and in vitro expressed Nup98 in the absence or presence of either MBP or MBP fused to M3 (Fig. 4). Most of the binding of Nup98 to Kapβ2 was abolised in the presence of MBP alone (Fig. 4). These results indicate that Nup98 is able to compete for the substrate-binding site on Kapβ2. Thus, when Kapβ2 with its bound cargo reaches the nuclear side of the NPC, Nup98 might release the cargo. In order to complete the import cycle, Kapβ2 would then need to be released from Nup98. Since Ran is known to regulate the assembly and disassembly of nuclear transport complexes, we sought to determine the effect of Ran on the binding of Kapβ2 to Nup98.
Binding of Kapβ2 to Nup98 Is Regulated by Ran—In vitro binding assays were performed with immobilized GST-Kapβ2 and in vitro expressed Nup98 in the absence or presence of Ran loaded with either GDP or GMPPNP (a non-hydrolyzable GTP analogue). As shown in Fig. 5, RanGMPPNP inhibited the binding of Kapβ2 to Nup98, whereas RanGDP had no such effect. These results indicate that RanGTP could dissociate Kapβ2 from Nup98 at the nucleoplasmic side of the NPC. Thus, during import, the Kapβ2-substrate complex could be dissociated by binding of Kapβ2 to the M9-like sequence of Nup98, leading to the release of substrate. The next step would be to dissociate Kapβ2 from Nup98, which can be accomplished by RanGTP. However, Ran is imported through the NPC primarily in its GDP-bound form, and conversion of RanGDP to RanGTP can only be accomplished by RanGEF (45, 50). RanGEF is known to be present in the nuclear interior in association with chromatin (51) but has not been shown in association with the NPC. It was therefore of interest to determine whether RanGEF is also found at the NPC where it would catalyze the conversion of RanGDP to RanGTP in order to release Kapβ2 from Nup98.

RanGEF Is Associated with the NPC—Isolated rat liver nuclear envelopes were probed with antibodies to RanGEF followed by secondary gold-conjugated antibody. RanGEF was found associated with both sides of the NPC (Fig. 6A). On the cytoplasmic side, the distribution of gold particles gave a mean distance of 42.9 nm from the midplane of the nuclear envelope (n = 171). On the nucleoplasmic side, the mean distance was 39.6 nm (n = 164) (Fig. 6B). Interestingly, the localization of RanGEF at the nucleoplasmic side of the NPC coincides with the previously determined localization of Nup98 (mean distance of 39 nm) (10).

To demonstrate further the presence of functional RanGEF at the NPC, we tested our nuclear envelope preparation for RanGEF exchange activity. Ran was labeled with [α-32P]GTP and incubated with excess unlabeled GDP in the absence or presence of isolated rat liver nuclear envelopes or recombinant RanGEF (Fig. 7). Exchange activity results in replacement of labeled nucleotide by unlabeled nucleotide and is measured as a decrease in Ran-associated radioactivity. As shown in Fig. 7, nuclear envelopes exhibited exchange activity comparable to that of recombinant RanGEF. These results demonstrate that functional RanGEF is present at the NPC.

Nup98 Contains a RanGEF-binding Domain—Since the sub-localization of RanGEF on the nuclear side of the NPC coincides with that of Nup98 (Fig. 8), we tested whether RanGEF binds to Nup98. Immobilized GST-RanGEF bound to ivNup98 but not to Nup96 (Fig. 8A), indicating that the binding is specific. To determine whether the binding is direct, immobilized recombinant GST-Nup98 was incubated with recombinant RanGEF. RanGEF bound to Nup98 in this assay as well (Fig. 8B), demonstrating that the two proteins interact directly. RanGEF did not bind to immobilized GST alone (Fig. 8B) confirming that the binding of RanGEF to Nup98 is specific.

In order to map the RanGEF-binding site on Nup98, in vitro
binding assays were carried out with immobilized GST-RanGEF and in vitro expressed wild-type Nup98 or a series of truncated mutants of Nup98 (Fig. 8C, see also schematics in Fig. 3). These results showed that the region between residues 316–405 of Nup98 is necessary for RanGEF binding. Thus the RanGEF-binding site is immediately downstream of the Kap2-binding site on Nup98, and nucleotide exchange at this location would make RanGTP immediately available for dissociation of Kap2 from Nup98.

**Discussion**

Nuclear import of macromolecules depends on interactions among import substrates, karyopherins, other soluble transport factors, and nucleoporins. Despite the advances made during the last decade in identifying the major mediators of nuclear import, much remains to be elucidated about the actual steps that result in the translocation of import substrates through the NPC and their delivery to their destinations inside the nucleus.

Different karyopherins may utilize different mechanisms for import and may selectively interact with different subsets of nucleoporins (10–12). Recent studies on the molecular mechanisms of import have revealed differences between the import mechanisms of Kapβ1 and Kapβ2. Kapβ1-mediated import requires RanGTP, and recycling of Kapβ1 requires GTP hydrolysis (54, 59). On the nuclear side, RanGTP binding to Kapβ1 seems to be required for release of import substrate into the nucleus (60). On the cytoplasmic side of the NPC, Kapβ1 and RanGTP form a ternary complex with the RanBP1 homologous domains of Nup358. Interaction of Kapα and cNLS with this complex stimulates GTP hydrolysis by RanGAP1 allowing the reinitiation of import of a cNLS-bearing substrate in permeabilized cells (54).

The situation is different with Kapβ2, which binds its import substrate directly. There is evidence that in the presence of equimolar concentrations of Kapβ2 and its import substrate, Ran is not required for nuclear accumulation of the substrate in permeabilized cell assays (49). However, when Kapβ2 is provided in smaller amounts, necessitating repeated rounds of import for delivery of substrate into the nucleus, RanGTP becomes a requirement for import. These data suggest the following: (i) that substrate release from the nuclear side of the

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**Fig. 3.** Kapβ2 binds to the FG-containing repeat region at the N terminus of Nup98 that includes the M9-like sequence and a downstream region (residues 1–316). A, schematic representation of the wild-type and truncated mutants of Nup98. Binding activity is indicated on the right based on the ratio between the bound and unbound fractions shown in B. B, wild-type and truncated mutants of Nup98 were obtained by transcription/translation in a reticulocyte in the presence of [35S]methionine and incubated with immobilized recombinant GST-Kapβ2 (1 μg) as described under “Experimental Procedures.” Bound and unbound fractions were analyzed by SDS-PAGE followed by autoradiography.

**Fig. 4.** Interaction of Nup98 with Kapβ2 is inhibited by MBP-M3. Nup98 was transcribed and translated in vitro in the presence of [35S]methionine, and incubated with immobilized recombinant GST-Kapβ2 (1 μg), in the absence or presence of MBP (20 μg) or MBP-M3 (20 μg). Bound and unbound fractions were analyzed by SDS-PAGE and autoradiography.

**Fig. 5.** Kapβ2 binding to Nup98 is inhibited by RanGMPPNP. Nup98 was transcribed and translated in vitro in the presence of [35S]methionine and incubated with immobilized recombinant GST-Kapβ2 (1 μg), in the absence or presence of RanGMPPNP (15 μg) or RanGDP (15 μg). Bound and unbound fractions were analyzed by SDS-PAGE and autoradiography.
NPC does not require Ran, and (ii) that RanGTP is required for recycling of Kap2. The data presented here provide mechanisms for both of these observations. As discussed below, the Ran-independent release of substrate probably occurs through competition of the Nup98 M9-like sequence with the M9 sequence on the import substrate. As binding of RanGTP to Kap2 results in dissociation of Kap2 from Nup98, recycling of Kap2 could occur through the local production of RanGTP by RanGEF that is bound to Nup98.

Nup98 and its yeast homolog, N-Nup145, have been implicated in RNA export pathways (20, 61–63). In this study, we have analyzed the role of Nup98 in import, specifically in the disassembly of Kap2 complexes at the nucleoplasmic side of the NPC and the recycling of Kap2. We have shown that Kap2 binds to the N-terminus of Nup98 at a site that contains an hnRNP A1 M9-like sequence. The M9-like sequence of Nup98 and M9 competed for binding to Kap2 indicating that binding of Kap2 to Nup98 would result in release of substrate from the nucleoplasmic side of the NPC once the import complex arrived there. A similar reaction may occur on Nup153 as well. An M9-like sequence has been described on Nup153 that could release M9 substrates from Kap2 (58). These M9-like sequences are likely to bind to the substrate-binding site of Kap2. However, the Kap2-binding sites in both Nup98 (Fig. 3) and Nup153 (58) extend beyond the M9-like sequence. Thus the interactions of Kap2 with these nucleoporins may not be limited to its substrate-binding site. The Kap2-Nup98 complex thus formed would be dissociated by RanGTP allowing the recycling of Kap2 for further rounds of import. We have shown that RanGEF is associated with both sides of the NPC, and we identified a RanGEF-binding site on Nup98 immediately C-
terminal to the Kapβ2-binding site. This binding is likely to be physiologically significant since we have shown that the localization of RanGEF at the nuclear side of the NPC coincides with that of Nup98 (10), and we demonstrated nucleotide exchange with isolated nuclear envelopes (Figs. 7 and 8). Nup98-associated RanGEF would catalyze the conversion of RanGDP to RanGTP which would then bind to Kapβ2 and dissociate it from Nup98 (Fig. 9).

Our finding that functional RanGEF is associated with the NPC may have more general implications for nuclear import pathways involving nucleoporins and karyopherins other than Nup98 and Kapβ2. It indicates that nucleotide exchange on Ran may occur at the NPC as an integral part of the transport process rather than being confined to the nuclear interior as previously thought. Previous data have been suggestive of the occurrence of nucleotide exchange on Ran at the NPC during Kapβ1-mediated import (60, 64). The binding of Kapβ1 to the nuclear side of the NPC is inhibited by RanGTP (65). Consistent with this finding, the binding of yeast Kapβ1 to the nucleoplasmic yeast nucleoporin Nup1 is inhibited by RanGTP (46). Furthermore, the binding of both Kapβ1 and Kapβ2 to Nup153 is inhibited by RanGTP (58). Thus, RanGTP is involved in the release of more than one karyopherin from nucleoporins at the nuclear side of the NPC. Further investigation is needed to determine whether these nucleoporins contain RanGEF-binding sites similar to Nup98. An alternative possibility is that Nup98-bound RanGEF provides the high local concentration of RanGTP needed for the release of karyopherins from other nucleoporins at the nuclear side of the NPC. Although RanGTP is required for recycling of Kapβ2 for repeated rounds of import, binding of RanGTP to Kapβ2 prevents the binding of import substrate (47). Therefore, the Kapβ2-RanGTP complex would probably need to be dissociated before further rounds of import can occur. The exact site and mechanism by which this is accomplished remain to be elucidated.

A role for RanGEF has also been proposed in the disassembly of nuclear export complexes, which presumably would occur at the cytoplasmic side of the NPC (66). In vitro studies with yeast proteins have suggested a role for RanGEF in disassembly of export complexes involving the nucleoporin Nup42 (66). Consistent with this notion, Nup42 has been recently localized to the cytoplasmic side of the NPC where disassembly of export complexes would be expected to occur (67). Our data (Fig. 6) provide evidence that RanGEF is indeed associated with the cytoplasmic side of the NPC. The mechanism of this association and the exact nucleoporin(s) involved remain to be determined. However, since it has been previously shown that RanGEF can form a ternary complex with RanBP1 (68), it is possible that a similar complex may form on the RanBP1-homologous domains of Nup358 at the cytoplasmic fibrils of the NPC. Indeed, the distance of a major portion of NPC-bound RanGEF on the

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**Fig. 8. Interaction of Nup98 with RanGEF. A**, Nup98 and Nup96 were transcribed and translated in vitro in the presence of [35S]methionine and incubated with immobilized recombinant GST-RanGEF (1 μg). Bound and unbound fractions were analyzed by SDS-PAGE and autoradiography. **B**, bacterially expressed immobilized GST-Nup98 or GST were incubated with recombinant RanGEF, and bound and unbound fractions were visualized by SDS-PAGE and Coomassie Brilliant Blue staining. **C**, wild-type (wt) and truncated mutants of Nup98 were in vitro transcribed and translated in the presence of [35S]methionine and incubated with immobilized recombinant GST-RanGEF (1 μg). A schematic representation of these proteins is shown in Fig. 3. Bound and unbound fractions were analyzed by SDS-PAGE followed by autoradiography.

**Fig. 9. Model for the role of Nup98 in Kapβ2-mediated nuclear import.** See text. S = import substrate; GEF = RanGEF.
cytoplasmic side is consistent with binding to Nup358 (15, 69).

Finally, the binding of RanGEF to the NPC may play a role in its import into the nucleus. It has recently been shown that RanGEF can be imported into the nucleus independently of any added soluble factors (70). This import may be mediated by the direct binding of RanGEF to nucleoporins. Kapβ1, Kapβ2, and importin-β have similarly been shown to enter the nucleus independently of added transport factors (71–73), a phenomenon that may also be mediated by the direct binding of these karyopherins to nucleoporins.

In summary, we have demonstrated that RanGEF is bound to a site on Nup98 that is immediately adjacent to an M9-like site. The high local concentrations of RanGTP generated by RanGEF combined with the M9-like sequence are likely to cooperate in the disassembly of Kapβ2 import complexes at the nuclear side of the NPC.

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In summary, we have demonstrated that RanGTP is bound to a site on Nup98 that is immediately adjacent to an M9-like site. The high local concentrations of RanGTP generated by RanGEF combined with the M9-like sequence are likely to cooperate in the disassembly of Kapβ2 import complexes at the nuclear side of the NPC.

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