Accelerating the Rate of Disassembly of Karyopherin-Cargo Complexes

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Transport of macromolecules across the nuclear pore complex (NPC) occurs in seconds and involves assembly of a karyopherin-cargo complex and docking to the NPC, translocation of the complex across the NPC via interaction with nucleoporins (Nups), and dissociation of the complex in the nucleoplasm. To identify rate-limiting steps in the Kap95p-Kap60p-mediated nuclear import pathway of Saccharomyces cerevisiae, we reconstituted key intermediate complexes and measured their rates of dissociation and affinities of interaction. We found that a nuclear localization signal-containing protein (NLS-cargo) dissociates slowly from Kap60p monomers and Kap60p-Kap95p heterodimers with half-lives (t1/2) of 7 and 73 min, respectively; that Kap60p and Kap60p-NLS-cargo complexes dissociate slowly from Kap95p (t1/2 = 36 and 73 min, respectively); and that Kap95p-Kap60p-NLS-cargo complexes and Kap95p-Kap60p heterodimers dissociate rapidly from the nucleoprotein Nup1p (t1/2 ≤ 21 s) and other Nups. A search for factors that accelerate dissociation of the long-lived intermediate revealed that Nup1p and Nup2p accelerate 16- and 19-fold the rate of dissociation of NLS-cargo from Kap60p. We suggest that Nup1p and Nup2p accelerate 16- and 19-fold the rate of dissociation of NLS-cargo from Kap60p-Kap95p heterodimers; that Gsp1p-GTP accelerates ≥ 447-fold the rate of dissociation of Kap60p-NLS-cargo from Kap95p; and that Nup2p and the Cse1p-Gsp1p-GTP complex independently accelerate ≥ 22- and ≥ 39-fold the rate of dissociation of NLS-cargo from Kap60p.

Nucleocytoplasmic protein transport across nuclear pore complexes (NPCs) proceeds at the rate of 1–1000 molecules/NPC/second (1–3) and requires the assembly and disassembly of several multiprotein intermediates (4). In general, mobile receptors termed karyopherins (or Kaps; these include importins and exportins, and transportins) bind the nuclear localization signal (NLS) or nuclear export signal (NES) of a cargo molecule and interact with nucleoporins (or Nups); nuclear pore complex proteins (to mediate translocation across the NPC. In the case of the Kap95p-Kap60p heterodimer of Saccharomyces cerevisiae, Kap60p (Srp1p) binds the NLS of a cargo molecule (NLS-cargo), and Kap95p tethers the Kap60p-NLS-cargo complex to Nups (docking), mediating translocation across the NPC via an unknown mechanism (see Fig. 1). Upon entering the nucleus, the NLS-cargo is released from Kap60p, and Kap95p and Kap60p are recycled separately to the cytoplasm for further rounds of import (5, 6).

Kap95p-Kap60p heterodimers can deliver NLS-cargos to the nucleoplasm against a concentration gradient of “free” cargo molecules (7), implying the existence of a mechanism at the NPC that imparts directionality to nuclear import. Structural features of the NPC may bias the direction of movement of Kap95p-Kap60p-NLS-cargo complexes within the NPC. In S. cerevisiae, the nuclear pore complex is composed of ~30 distinct nucleoporins (8). A subset of the Nups (FG Nups) contains repeats of the dipeptide motif FG, which bind Kaps (9, 10). Each of the 13 FG Nups of yeast is present in eight or more copies per NPC, and together FG Nups may contribute up to 200 docking sites for karyopherins within the NPC. Eight FG Nups are distributed symmetrically within the NPC (e.g. Nup100p and Nup116p), whereas the rest are localized exclusively to either the cytoplasmic fibers (Nup42p and Nup159p) or the nuclear basket structure (Nup1p, Nup2p, and Nup60p) (8, 11, 12). In vivo, the FG Nups of the nuclear basket (Nup1p, Nup2p, and Nup60p) are required for efficient Kap95p-Kap60p-mediated nuclear import (12–15). These same three Nups, plus the exportin Cse1p in complex with Gsp1p-GTP, are also required for efficient nuclear export of Kap60p in vitro (11, 12, 14–16).

The directionality of karyopherin-mediated transport is also governed by the Gsp1p GTPase (the Ran GTPase in vertebrates) (4). Binding of Gsp1p-GTP to importins (karyopherins dedicated to nuclear import) disrupts their ability to bind cargoes and Nups and thus terminates nuclear import reactions in the nuclear basket structure of the NPC or in the nucleoplasm (9, 17, 18). Conversely, exportins (karyopherins dedicated to nuclear export) bind cargoes and Gsp1p-GTP cooperatively to initiate export reactions in the nucleoplasmic side of the NPC (19, 20). Export reactions are terminated when Yrb1p stimulates dissociation of Gsp1p-GTP from exportins, and Rna1p stimulates the hydrolysis of GTP by Gsp1p (4). Because Rna1p is localized to the cytoplasm of yeast (21) and the Gsp1p guanine nucleotide exchange factor (Prp20p) is localized to the nucleoplasm (22, 23), a steep concentration gradient of Gsp1p-GTP across the NPC likely exists.

Although mechanistic details of Kap95p translocation across the NPC are lacking, it is clear that Kap95p orthologues in vertebrates (importin β and transportin) move at a very rapid rate through the NPC (1–1000 molecules/NPC/second) (1–3). It follows that each intermediate step in the nuclear import path-
The generation of Nup1p/H9004 N (amino acids 332-1076), which is missing the NH2 terminus of a chimera between GST and the maltose-binding protein (MBP). Recombinant proteins were expressed in BL21 Codon Plus (Novagen) and were purified on glutathione-coated beads. The GST-Nup, -Kap, or -NLS-cargo was incubated in batch with glutathione-Sepharose beads (1-2 μg of GST fusion/10 μl of packed beads; Amersham Biosciences) in 1 ml of binding buffer for 15 min at 4°C. The beads were collected by centrifugation at 2,000 × g for 30 s and washed six times by resuspension in 0.5 ml of binding buffer and sedimentation as before. Two washes were done at room temperature and contained 100 μM ATP to remove any E. coli heat shock proteins that bound to the GST chimeras. Washed collected beads were resuspended in a 50% slurry, and the bead slurry was aliquoted in 0.1%, and aliquots were frozen in liquid nitrogen and stored at −70°C. His-tagged Gsp1p was purified and charged with GTP as described previously (9, 17).

Solution Binding Assay—All assays were performed using recombinant proteins in binding buffer (20 mM Hepes, pH 6.8, 150 mM KOAc, 2 mM Mg(OAc)2, and 2 mM DTT). Peak fractions containing the purified karyopherin, nucleoporin, or NLS-cargo were pooled. Two washes were added to 0.1%, and aliquots were frozen in liquid nitrogen and stored at −70°C. His-tagged Gsp1p was purified and charged with GTP as described previously (9, 17).

Equilibrium Affinity Assay—Protein affinities were conducted as described previously (14). Purified Kap95p and Kap60p were phosphorylated at their NLS site using bovine heart kinase and [γ-32P]ATP (PerkinElmer Life Sciences), as described in the Amersham Biosciences GST handbook. GST-Kap60p, GST-Nup1pAN, and GST-NLS-cargo were immobilized separately on beads and incubated with increasing amounts of radiolabeled Kap95p or Kap60p. The GST-Nup1pAN was immobilized separately on beads and incubated with increasing amounts of radiolabeled Kap95p or Kap60p at room temperature in 20 mM Hepes, pH 6.8, 150 mM KOAc, 2 mM Mg(OAc)2, 1 mM DTT, 0.1% Tween 20, protease inhibitors (leupeptin, pepstatin, aprotinin, and phenylmethylsulfonyl fluoride), and 1 mg/ml BSA. After incubation, beads were washed quickly, and bound radiolabeled Kap95p or Kap60p was determined by counting in a scintillation counter.
beads were quantified by liquid scintillation. Binding curves were fit to the data using GraphPad Prism™ software (Biosoft).

**Molecular Dissociation Assay**—Dissociation rates were assayed using purified radiolabeled proteins in the bead-based solution binding assay. Kap95p and Kap60p were radiolabeled as above. GST-Kap60p, GST-Nup1pΔN, and GST-NLS-cargo were immobilized separately on beads and incubated for 2 h with radiolabeled Kap95p or Kap60p at room temperature in 20 mM Hapes, pH 6.8, 150 mM KOAc, 2 mM Mg(OAc)2, 1 mM DTT, 0.1% Tween 20, and 10 mg/ml BSA. Unlabeled Kap95p or Kap60p were also present when indicated. 12 μl of the bead mix was diluted into 1.2 ml of 20 mM Hapes, pH 6.8, 150 mM KOAc, 2 mM Mg(OAc)2, 1 mM DTT, 0.1% Tween 20, and 10 mg/ml BSA containing various concentrations of competitor proteins. Beginning 10 s after dilution, beads were flash-collected on filters using a vacuum manifold. Bound radiolabeled proteins were eluted with 1% SDS and quantified by scintillation counting. Single exponential dissociation curves were fit to the data using GraphPad Prism™ software (Biosoft). Half-lives of complexes ($t_{1/2}$) were calculated using the equation $t_{1/2} = \ln 2/k_{off}$. Mean residence time was calculated using the equation $1/k_{off}$. Association rate constants were calculated using the equation $k_{on} = K_D/k_{off}$.

**RESULTS**

There is ample cell biological and genetic evidence that the nucleoporins Nup1p and Nup2p, the GTPase Gsp1p, and the exportin Cse1p perform essential functions in the Kap95p-Kap60p-mediated nucleocytoplasmic transport pathway, and there is evidence of biochemical interactions among subsets of these proteins (9, 11, 12, 14–17, 24–26). However, the basic mechanism underlying the function of these proteins in the pathway is not known. Specifically, it has not been resolved whether these proteins function in a passive manner to modulate the interaction of Kap60p and/or Kap95p with other proteins or whether they function in an active manner to trigger allosteric mechanisms in Kap60p and/or Kap95p that accelerate capture or release of bound partners (for example see Fig. 5A). Here we addressed this issue directly by conducting a quantitative analysis of the dynamics of assembly and disassembly of key nuclear import intermediates.

An NLS-cargo molecule was constructed as a fusion of the NLS of *S. cerevisiae* Cbp80p (an mRNA cap-binding protein; amino acids 1–30) and the MBP. We chose the NLS of Cbp80p because it may be the highest affinity endogenous NLS recognized by Kap60p in yeast. This is based on the observation that Cbp80p is one of only two proteins in yeast extracts that remain bound to Kap60p when all other proteins have been removed by extraction (27) (data not shown). The second protein is Nup2p.

Nup1p was chosen for this analysis because (i) it binds Kap95p-Kap60p heterodimers with the highest affinity in comparison to other FG Nups tested (9) (data not shown), (ii) it functions in the Kap95p-Kap60p transport pathway *in vivo* (12, 26), and (iii) it resides in the nuclear basket structure of the yeast NPC (8, 12) where disassembly of import complexes is presumed to occur. Indeed, Nup1p may function as one of the last “stepping stones” (docking sites) in the path of Kap95p-Kap60p-mediated transport (Fig. 1). In addition to its serving as a stepping stone for transport, we demonstrate here that Nup1p accelerates the rate of dissociation of NLS-cargo from Kap60p-Kap95p heterodimers. We used a version of Coomassie Blue stain. B, reconstitution of the Kap60p-Kap95p heterodimer. GST-Kap60p immobilized on beads (1 μg/10 μl of beads; or 1.1 μM within the bead volume) was mixed with purified Kap95p (1 μg; final concentration of 263 nM). After 2 h at 4 °C, reactions were processed and analyzed as described for A. C, reconstitution of karyopherin-nucleoporin complexes in the presence and absence of NLS-cargo. GST-Nup1pΔN immobilized on beads (1 μg/10 μl of beads; 0.9 μM within the bead volume) was mixed with purified Kap60p, Kap95p, and NLS-cargo (MBP-NLS/Cbp80p) as indicated (1 μg each; final concentrations of 416, 273, and 566 nM, respectively). After 2 h at 4 °C, the reactions were processed and analyzed as described for A.

**FIG. 2.** Reconstitution of key intermediate complexes in the Kap95p-Kap60p-mediated nuclear import pathway. The diagrams depict reconstituted intermediate complexes. The gray tether symbols mark the immobilized protein used in each case. A, reconstitution of the Kap60p-NLS-cargo complex in the presence and absence of Kap95p. GST-MBP-NLS/Cbp80p (GST-NLS-cargo) immobilized on glutathione-coated Sepharose beads (1 μg/10 μl of beads; 1.4 μM within the bead volume) was mixed with purified Kap95p and Kap60p as indicated (1 μg each for final concentrations of 417 and 263 nM, respectively). After 2 h at 4 °C, beads were collected by centrifugation, and the supernatant containing unbound proteins was removed. Beads were washed twice, and bound proteins were extracted with SDS. Proteins in the bound and unbound fractions were resolved by SDS-PAGE and visualized with Coomassie Blue stain. B, reconstitution of the Kap60p-Kap95p heterodimer. GST-Kap60p immobilized on beads (1 μg/10 μl of beads; or 1.1 μM within the bead volume) was mixed with purified Kap95p (1 μg; final concentration of 263 nM). After 2 h at 4 °C, reactions were processed and analyzed as described for A. C, reconstitution of karyopherin-nucleoporin complexes in the presence and absence of NLS-cargo. GST-Nup1pΔN immobilized on beads (1 μg/10 μl of beads; 0.9 μM within the bead volume) was mixed with purified Kap60p, Kap95p, and NLS-cargo (MBP-NLS/Cbp80p) as indicated (1 μg each; final concentrations of 416, 273, and 566 nM, respectively). After 2 h at 4 °C, the reactions were processed and analyzed as described for A.
**Figure 3.** Equilibrium affinities of key nuclear import intermediates. In the diagrams, the gray tether symbols mark the immobilized protein, and the radioactivity symbols mark the radiolabeled protein. Each data point was performed in duplicate, and error bars represent S.E. The curves are representative of duplicate experiments. A. Affinity of Kap60p towards NLS-cargo. GST-NLS-cargo-coated beads (13 ng total; 50 nM within the bead volume) were incubated with various concentrations of radiolabeled Kap60p for 2 h at 25 °C in binding buffer with 1 mg/ml BSA and protease inhibitors. Beads were collected by sedimentation and washed once, and the amount of radiolabeled Kap60p bound was quantified in a scintillation counter. The dissociation constant (KD) of the Kap60p-NLS-cargo complex was calculated as described under “Experimental Procedures.” The results are plotted as a fraction of maximal radiolabeled Kap60p bound versus Kap60p concentration added. Lower concentrations of immobilized GST-NLS-cargo produced identical results. B. Affinity of Kap60p towards NLS-cargo in the presence of Kap95p. Kap95p and Kap60p were labeled with 32P at an engineered tag in their NH2 termini, which does not interfere with their functioning (see “Experimental Procedures”) (28). After 2 h at 25 °C, beads were collected by centrifugation, and the amount of bound radiolabeled karyopherin was quantified by liquid scintillation. The results are shown in Fig. 3; the diagrams depict the immobilized protein bound to cross-hatched surface, the radiolabeled ligand marked by the radioactivity symbols mark the radiolabeled protein. Each data point was performed in duplicate, and error bars represent S.E. The curves are representative of duplicate experiments. A. Affinity of Kap60p towards NLS-cargo. 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TABLE I

Molecular dynamics of nuclear import intermediates

| Intermediate complex in nuclear import | Measured affinity (KD) (pM) | Measured dissociation rate constant (k off) (s^-1) | Calculated association rate constant (k on) (s^-1) | Half-life of complex (min) | Mean interaction time (s) |
|---------------------------------------|-----------------------------|-----------------------------------------------|-----------------------------------------------|---------------------------|--------------------------|
| *Kap60p · NLS-cargo                  | 2800                        | 1.8 × 10^-3                                   | 6.4 × 10^5                                   | 394 s                     | 556 s                    |
| Kap95p · *Kap60p · NLS-cargo          | ≤ 149                       | 1.6 × 10^-4                                   | ≥ 1.1 × 10^6                                 | 73 min                    | 104 min                  |
| *Kap60p · NLS-cargo                  | ND                          | 1.6 × 10^-4                                   | ND                                           | 73 min                    | 104 min                  |
| *Kap95p · Kap60p                     | ≤ 153                       | 3.2 × 10^-4                                   | ≥ 2.1 × 10^6                                 | 36 min                    | 52 min                   |
| Nup1pΔN · *Kap95p · Kap60p            | ≤ 51                        | ≥ 3.3 × 10^-2                                 | ≥ 6.5 × 10^8                                 | ≤ 21 s                    | ≤ 30 s                   |
| Nup1pΔN · *Kap60p · NLS-cargo        | 400                         | ≥ 3.3 × 10^-2                                 | ≥ 8.3 × 10^7                                 | ≤ 21 s                    | ≥ 30 s                   |

activity symbol, and other proteins present in each incubation. Not shown is bovine serum albumin, which was present at 1 mg/ml in these reactions.

Kap60p bound to an immobilized NLS-cargo with KD = 2.8 nM (Fig. 3A and Table I). This affinity is similar to the affinity of Kap60p for the SV40 T-antigen NLS (KD = 2 nM) (29). Notably, the presence of Kap95p increased by ≥18-fold the affinity of Kap60p for the NLS-cargo (from KD = 2.8 nM to KD ≤ 149 pM) (Fig. 3, A and B). This is consistent with previous results (17, 30) and is likely due to the binding of Kap95p to the IBB domain of Kap60p, which otherwise serves as an intramolecular inhibitor of NLS binding (31). The affinity value obtained for the interaction between Kap95p-Kap60p and NLS-cargo (KD ≤ 149 pM) is near the limit of detection of our assay. The actual KD may be lower (indicated by the ≤ symbol) because reduction of the concentration of immobilized NLS-cargo yielded a lower apparent KD (data not shown). However, loss of signal at reduced concentrations of Kap95p-Kap60p prevented unambiguous quantitation. In such cases, we have reported the most accurate value we could measure.

Kap95p bound immobilized Kap60p with KD ≥ 153 pm (Fig. 3C and Table I). This value was near the limit of detection of our assay (as explained above), and so the actual KD for the Kap95p-Kap60p complex may be lower. The interaction of Kap95p with Kap60p is often referred to as a typical karyopherin-cargo interaction because Kap95p binds an NLS-like sequence in Kap60p (the IBB domain). However, it is possible that Kap60p modulates Kap95p function in ways that other cargoes that bind Kap95p directly do not.

Kap95p-Kap60p-NLS-cargo complexes bound immobilized Nup1pΔN with KD = 400 pm (Fig. 3D and Table I). Kap95p-Kap60p heterodimers (without NLS-cargo) bound Nup1pΔN with KD ≤ 51 pm (Fig. 3E). The latter value was at the limit of detection of our assay (as explained above), and thus the reported number is an upper bound on the actual KD. As both Kap95p and Kap60p can bind independently to Nup1pΔN (Fig. 2C), in principle both karyophersins could mediate the interaction when binding in heterodimeric form. This may explain the exceptionally high affinity of Kap95p-Kap60p toward Nup1pΔN. The presence of an NLS-cargo bound to Kap60p-Kap95p lowered by ≥8-fold the affinity of Kap95p-Kap60p toward Nup1pΔN (from KD ≤ 51 pm to KD ≥ 400 pm (Fig. 3, D and E; Table I). Because it is possible that different NLSs have different effects on Kap60p-Kap95p interactions with Nups or other proteins, we conducted experiments in the absence and presence of NLS-cargo to offer a comparison between cargo-bound and cargo-free Kap95p-Kap60p.

The nuclear import intermediates examined above exhibit high affinities of interaction. These affinities reflect the ratio between the dissociation rate constant (k off) and the association rate constant (k on) of the interacting pairs (KD = k off/k on).

Assuming typical association rate constants for protein-protein interactions (10^7–10^8 M^-1 s^-1) (32), the high affinities of the nuclear import intermediates measured here predict interactions on a time scale of many seconds to minutes. However, a high affinity of binding is not necessarily incompatible with fast rates of dissociation. In cases in which proteins can bind each other at rates that approach the limit set by molecular diffusion (k on = 10^-6-10^-10 M^-1 s^-1) (32), a molecular interaction of very high affinity (e.g. KD = 0.5 nM) could dissociate with a half-life of less than 2 s.

Dissociation Rate Constants of Nuclear Import Intermediates—The solution binding assay used above was modified to quantify molecular dissociation rates between Nups, Kaps, and NLS-cargo (Fig. 4). Immobilized NLS-cargo, Kap60p, or Nup1p was incubated with radiolabeled Kap95p or Kap60p as depicted in the diagrams. After 2 h at 25°C, binding reactions were diluted 100-fold into buffer containing unlabeled Kap95p, Kap60p, or NLS-cargo as competitors. At intervals of seconds or minutes, aliquots from the diluted mix were removed and their beads flash-collected in a manifold filter. The amount of radiolabeled protein bound to beads was quantified by liquid scintillation.

The inclusion of unlabeled proteins as competitors in the molecular dissociation assays played an important role. Excess unlabeled Kaps prevented rebinding of radiolabeled Kaps to sites in immobilized proteins that become available when radiolabeled Kaps dissociate following the dilution step. The amount of competitor needed to prevent significant rebinding of radiolabeled Kaps to immobilized proteins in each case was determined experimentally by conducting dissociation assays in the presence of increasing concentrations of unlabeled competitor until the apparent rate of dissociation showed no further change (Fig. 4).

The dissociation of radiolabeled Kap60p from immobilized NLS-cargo was slow (k off = 1.8 × 10^-3 s^-1) (Table I) with an apparent t1/2 of the complex of 394 s in the presence of saturating amounts of unlabeled competitor Kap60p (200 nM) (Fig. 4A). Increasing the concentration of competitor Kap60p from 200 nM to 1.6 µM caused no further increase in the apparent rate of dissociation of radiolabeled Kap60p (Fig. 4A and data not shown). Therefore, 200 nM unlabeled Kap60p was sufficient to prevent significant rebinding of radiolabeled Kap60p to immobilized NLS-cargo. Under such conditions of no rebinding, the rate measured for Kap60p dissociation from NLS-cargo is accurate.

Kap95p reduced the rate of dissociation of Kap60p from NLS-cargo (Fig. 4B). In the presence of Kap95p, dissociation of radiolabeled Kap60p from immobilized NLS-cargo occurred 10 times more slowly (k off = 1.6 × 10^-4 s^-1) than in the absence of Kap95p (k off = 1.8 × 10^-3 s^-1), with a half-life of the complex of > 1 h (Fig. 4B and Table I). Inclusion of 200 or 800 nM unlabeled competitor Kap60p-Kap95p produced similar rates of dissociation, indicating that significant rebinding of radiolabeled Kap60p had been prevented. Thus, the rate measured for Kap60p-Kap95p dissociation from NLS-cargo is accurate.
FIG. 4. Rates of dissociation of nuclear import intermediates. The diagrams depict the immobilized Nups, Kaps, or NLS-cargo with a gray tether symbol and radiolabeled Kaps with a radioactivity symbol.

A, dissociation of NLS-cargo from Kap60p. GST-NLS-cargo-coated beads (0.3 μg; 80 nM within the bead volume) were incubated with 20 nM radiolabeled Kap60p for 2 h at 25 °C. Incubations were then diluted 100-fold into buffer containing 10 mg/ml BSA and additional proteins as indicated. At intervals beginning 10 s after dilution, beads in aliquots were flash-collected in a manifold filter. The amount of radiolabeled Kap remaining in the beads was determined by liquid scintillation. The half-life of complexes was calculated as described under “Experimental Procedures.” Each data point was performed in duplicate, and error bars represent S.E. For convenient comparisons, the first data point of each curve was normalized to 100%. The curves shown are representative of duplicate experiments.

B, dissociation of NLS-cargo from Kap60p·Kap95p. GST-NLS-cargo-coated beads (0.3 μg; 80 nM within the bead volume) were incubated with 200 nM Kap95p and 20 nM radiolabeled Kap60p for 2 h at 25 °C. Incubations were then diluted 100-fold into buffer containing 10 mg/ml BSA and additional proteins as indicated. Samples were processed and the results plotted as described in A. C, dissociation of Kap60p·Kap95p·NLS-cargo from Kap95p. GST-Kap60p-coated beads (0.5 μg; 80 nM within the bead volume) were incubated with 200 nM NLS-cargo and 20 nM radiolabeled Kap95p for 2 h at 25 °C. Incubations were then diluted 100-fold into buffer containing 10 mg/ml BSA and additional proteins as indicated. Samples were processed and the results plotted as described in A. D, dissociation of Kap95p from Kap60p. GST-Kap60p-coated beads (0.5 μg total; 80 nM within the bead volume) were incubated with 20 nM radiolabeled Kap95p for 2 h at 25 °C. Incubations were then diluted 100-fold into buffer containing 10 mg/ml BSA and additional proteins as indicated. Samples were processed and the results plotted as described in A.

E and F, dissociation of Kap95p·Kap60p from Nup1p1ΔN in the presence and absence of NLS-cargo. GST-Nup1p1ΔN-coated beads (0.1 μg total; 40 nM within the bead volume) were incubated with 3 nM radiolabeled Kap95p and 10 nM Kap60p with or without 40 nM NLS-cargo for 2 h at 25 °C. Incubations were then diluted 100-fold into buffer containing 10 mg/ml BSA and additional proteins as indicated. Samples were processed and the results plotted as described in A. The dissociation rate constant (k_{off}) may be greater than this value because higher concentrations of competitor revealed a larger dissociation rate constant.
Rapid Disassembly of Kap95p-Kap60p-NLS-Cargo Complexes

Radiolabeled Kap95p dissociated very slowly from immobilized Kap60p-NLS-cargo ($k_{off} = 1.6 \times 10^{-4} \text{ s}^{-1}$) with a half-life of 73 min (Fig. 4C and Table I). Increasing concentrations of competitor Kap95p up to 800 nM produced no further increase in the rate of dissociation of radiolabeled Kap95p from Kap60p-NLS-cargo, indicating that significant rebinding of radiolabeled Kap95p was prevented and that the rate of dissociation measured is accurate. In the absence of NLS-cargo, Kap95p dissociated from immobilized Kap60p twice as fast as when NLS-cargo was present (Fig. 4D and Table I). Increasing the concentration of unlabeled competitor Kap95p up to 800 nM produced no further increase in the apparent rate of dissociation of radiolabeled Kap95p from Kap60p. Thus the measured rate of Kap95p dissociation from Kap60p-NLS-cargo is also accurate.

Surprisingly, Kap95p-Kap60p-NLS-cargo complexes and Kap95p-Kap60p heterodimers dissociated very rapidly from Nup1pΔN with half-lives of ≤ 21 s (Fig. 4, E and F; Table I). Rapid dissociation occurred despite the very high affinity of Kap95p-Kap60p for Nup1pΔN in the presence and absence of NLS-cargo ($K_D = 400$ and ≤ 51 pm, respectively) (Fig. 3, D and E). Increasing the concentration of unlabeled competitors revealed an even faster dissociation of the Kap-Nup complexes, at rates beyond the limit of detection of our assay (data not shown). Thus, the reported ≤ 21 s half-lives for the interactions of Kap95p-Kap60p-NLS-cargo and Kap95p-Kap60p with Nup1pΔN represent the slowest dissociation rate exhibited by these complexes. Similar experiments using immobilized FG regions of Nup100p and Nup116p revealed even faster dissociation of Kap95p-Kap60p from those FG Nups (data not shown).

Accelerating Disassembly of Long-lived Nuclear Import Intermediates—Among the nuclear import intermediates reconstituted and characterized above, only the Kap-Nup complexes appear to spontaneously disassemble fast enough ($t_{1/2} \leq 21$ s) to serve as intermediates in a rapid nuclear import mechanism. In contrast, the dissociation of NLS-cargo from Kap60p-Kap95p ($t_{1/2} = 73$ min) and the dissociation of Kap95p from Kap60p-NLS-cargo ($t_{1/2} = 73$ min) were extremely slow and represent potentially rate-limiting steps for nuclear import in vivo. Factors may exist within the NPC or nucleoplasm that accelerate the dissociation of these long-lived complexes.

Gsp1p may function to accelerate molecular dissociation, as Gsp1p-GTP binding to Kap95p disrupts its association with Kap60p and with Kap60p-NLS-cargo complexes (17). Structural studies suggest that Ran-GTP (Gsp1p-GTP) may cause local conformational changes in importin β (Kap95p) that progressively loosen importin α (Kap60p) and lead to its release (33). However, direct evidence of such a mechanism has not been obtained. Previous studies did not distinguish between two possible mechanisms of Gsp1p-GTP function (see Fig. 5A): (i) a passive mechanism in which Gsp1p-GTP must wait for Kap95p to spontaneously dissociate from Kap60p before binding Kap95p (thus preventing its re-association with Kap60p); or (ii) an active mechanism in which Gsp1p-GTP binds Kap95p in Kap95p-Kap60p complexes forming a transient trimeric intermediate that triggers rapid release of Kap60p.

We tested whether Gsp1p-GTP could accelerate the rate of dissociation of Kap95p from Kap60p in the presence and absence of NLS-cargo (Fig. 5, B and C). The dissociation of radiolabeled Kap95p from immobilized Kap60p or Kap60p-NLS-cargo complex was monitored in the presence of increasing concentrations of Gsp1p-GTP as a possible effector or in the presence of excess unlabeled competitor Kap95p as a control. The amount of competitor needed to block rebinding of radiolabeled Kap95p was determined experimentally in Fig. 4. We expected to find either (i) no increase in rate of dissociation after addition of Gsp1p-GTP, consistent with a passive mechanism of Gsp1p function (Fig. 5A), or (ii) an increase in the rate of dissociation after addition of Gsp1p-GTP, consistent with an active mechanism for Gsp1p function (Fig. 5A). 200 nM Gsp1p-GTP stimulated 447-fold the apparent rate of dissociation of Kap95p from Kap60p-NLS-cargo complexes relative to the intrinsic rate of dissociation of this complex in the absence of Gsp1p-GTP (Fig. 5B; compare curves 1 and 3; Table II). In the absence of NLS-cargo, 50 nM Gsp1p-GTP stimulated the rate of dissociation of Kap95p from Kap60p by ≥ 65-fold (Fig. 5C and Table II). Greater concentrations of Gsp1p-GTP allowed radiolabeled Kap95p to dissociate even faster from immobilized Kap60p-NLS-cargo or Kap95p, at rates that were too fast to measure with our assay. The data are consistent with an active mechanism of Gsp1p function in which Gsp1p-GTP binds to Kap95p in a Kap95p-Kap60p-NLS-cargo complex (or in a Kap95p-Kap60p complex) and triggers an allosteric mechanism in Kap95p that causes rapid release of Kap60p (and its bound NLS-cargo) (17, 33). The concentration of Gsp1p-GTP in the yeast nucleoplasm is estimated at 1–10 μM (14). Thus, it is expected that Gsp1p-GTP will bind Kap95p and trigger the release of Kap60p-NLS-cargo at a rate much faster in vivo than that measured here in vitro.

Following the Gsp1p-GTP-mediated dissociation of Kap60p-NLS-cargo from Kap95p, the remaining Kap60p-NLS-cargo complex is stable and dissociates with a half-life of 394 s (Fig. 4A and Table I). It has been suggested that Nup2p promotes dissociation of NLS-cargo from Kap60p (12) and that Nup1p promotes dissociation of NLS-cargo from Kap95p-Kap60p heterodimers (17), but the distinctions between passive or active mechanisms of function have not been established experimentally. For Kap60p, may also promote dissociation of NLS-cargo from Kap60p because the binding of Cse1p and NLS-cargo to Kap60p is mutually exclusive (6). We therefore tested whether Cse1p, Nup1p, or Nup2p could accelerate the rate of dissociation of NLS-cargo from Kap60p.

First, we tested whether Cse1p-Gsp1p-GTP could accelerate release of NLS-cargo from Kap60p (Fig. 6A). The dissociation of radiolabeled Kap60p from immobilized NLS-cargo was monitored in the presence of increasing concentrations of Cse1p-Gsp1p-GTP as a possible effector or in the presence of excess unlabeled Kap60p as a control. The amount of competitor Kap60p needed to block significant rebinding of radiolabeled Kap60p was determined experimentally in Fig. 4A. Gsp1p-GTP is a co-factor for Cse1p function (6, 25) and does not bind Kap60p. 800 nM Cse1p-Gsp1p-GTP increased by ≥ 39-fold the rate of dissociation of Kap60p from NLS-cargo (from $k_{off} = 1.8 \times 10^{-3} \text{ s}^{-1}$ to ≥ 6.9 \times 10^{-2} \text{ s}^{-1}$; or $t_{1/2} = 394$ to ≤ 10 s) (Fig. 6A, compare curves 1 and 3; Table II). It is possible that higher concentrations of Cse1p-Gsp1p-GTP accelerate further the rate of dissociation of Kap60p from NLS-cargo, but our assay could not monitor faster rates. The data obtained are consistent with an active mechanism of Cse1p function whereby Cse1p (in a Cse1p-Gsp1p-GTP complex) binds Kap60p (in a Kap60p-NLS-cargo complex) to form a transient tetrameric intermediate that triggers an allosteric mechanism in Kap60p, which causes rapid release of the NLS-cargo (Fig. 6A, diagram). In this role, the exportin Cse1p functions as a nuclear import factor that dissociates NLS-cargo from the importin Kap60p.

The presence of both Cse1p and Gsp1p-GTP was required to accelerate the release of NLS-cargo from Kap60p (Fig. 6B). In the presence of either 200 nM Cse1p or 200 nM Gsp1p-GTP alone, the amount of radiolabeled Kap60p that remained bound to immobilized NLS-cargo after 5 min was unaffected in com-
comparison with a control incubation containing 200 nM unlabeled competitor Kap60p. This indicates that neither Cse1p nor Gsp1p-GTP alone could accelerate Kap60p dissociation from the NLS-cargo. However, when 200 nM Cse1p and 200 nM Gsp1p-GTP were combined, nearly all of the bound Kap60p dissociated from the NLS-cargo within 5 min (Fig. 6B). This demonstrates cooperation between Cse1p and Gsp1p in accelerating release of NLS-cargo from Kap60p, which is consistent with the finding that Cse1p binds Kap60p in the presence of Gsp1p-GTP (6, 25).

We next tested whether the nucleoporins Nup1p and Nup2p could accelerate disassembly of Kap60p-NLS-cargo complexes (Fig. 7). Dissociation of radiolabeled Kap60p from immobilized NLS-cargo was monitored as described above, in the presence
of Nup1pΔN or Nup2p as possible effectors or in the presence of competitor unlabeled Kap60p as a control. The concentration of competitor Kap60p sufficient to prevent significant rebinding of radiolabeled Kap60p was determined experimentally in Fig. 4A. 800 nM Kap60p accelerated 22-fold the disassembly of Kap60p-NLS-cargo complexes relative to the intrinsic rate of Kap60p-NLS-cargo dissociation (from \( k_{\text{off}} = 1.8 \times 10^{-3} \text{ s}^{-1} \) to \( t_{1/2} = 394 \text{ s} \) in Table I). In comparison, the concentration of Nup2p in the nuclear basket structure of the NPC is estimated to be \(-10 \mu\text{m} \) based on the approximate number (30) of Nup2p molecules/NPC (15) and the volume occupied by the nuclear basket structure where it resides. Together the data suggest that Nup2p binds Kap60p in Kap60p-NLS-cargo complexes, forming a trimeric intermediate that triggers an allosteric mechanism in Kap60p, which causes rapid release of NLS-cargo (Fig. 7, diagram). Binding of Nup2p to Kap60p is a prerequisite for the observed effect, as a mutant of Nup2p that does not bind Kap60p does not stimulate release of NLS-cargo from Kap60p (data not shown). Nup1pΔN also bound Kap60p directly (Fig. 2C), but it did not stimulate release of NLS-cargo from Kap60p. The addition of Nup1pΔN at concentrations ranging from 40 to 200 nM accelerated dissociation of NLS-cargo from Kap60p, but it did not stimulate release of NLS-cargo from Kap60p (Fig. 7, Table II).
800 nm caused no increase in the rate of dissociation of Kap60p-NLS-cargo complexes (Fig. 7 and data not shown).

Finally, we tested whether Nup1p and Nup2p could increase the rate of dissociation of NLS-cargo from Kap60pKap95p heterodimers (Fig. 8), as opposed to release of NLS-cargo from Kap60p monomers. Dissociation of Kap60pKap95p from an immobilized NLS-cargo was monitored in the presence of Nup1pΔN or Nup2p as possible effectors or in the presence of excess unlabeled competitor Kap60pKap95p as control. The amount of competitor Kap60pKap95p sufficient to prevent rebinding of radiolabeled Kap60pKap95p to immobilized NLS-cargo was determined experimentally in Fig. 4B. 40 nm Nup1pΔN caused a 16-fold increase in the dissociation rate of Kap95pKap60p from immobilized NLS-cargo (from \(k_{\text{off}} = 1.6 \times 10^{-4} \text{s}^{-1}\) to \(2.5 \times 10^{-3} \text{s}^{-1}\); or \(t_{1/2} = 4380\) to 276 s) (Fig. 8 and Table I). Likewise, 40 nm Nup2p caused a 19-fold increase in the rate of dissociation of Kap95pKap60p from the immobilized NLS-cargo (from \(k_{\text{off}} = 1.6 \times 10^{-4} \text{s}^{-1}\) to \(3 \times 10^{-3} \text{s}^{-1}\); or \(t_{1/2} = 4380\) to 228 s) (Fig. 8 and Table I). Higher concentrations of Nup2p or Nup1pΔN did not further stimulate the rate of Kap95pKap60p dissociation from the NLS-cargo (data not shown). The effect of Nup1pΔN and Nup2p on Kap95pKap60p-NLS-cargo complexes may involve a reaction in which Nup1pΔN and Nup2p bind directly to Kap95p, Kap60p, or both, forming a tetrameric intermediate that changes the structure of Kap60p to weaken its interaction with the NLS-cargo (Fig. 8, diagram). The ability to exert an allosteric effect on Kap95p may be unique to Nup1p and Nup2p, as other FG Nups such as Nup100p and Nup116p cannot accelerate the dissociation of NLS-cargo from Kap60pKap95p even when added at concentrations well above the affinity of Kap95pKap60p-NLS-cargo toward these FG Nups (data not shown).

**DISCUSSION**

We describe several molecular mechanisms by which slow, potentially rate-limiting steps in the Kap95pKap60p-mediated nuclear import pathway may be overcome in *S. cerevisiae*. We find that resident proteins of the nuclear basket structure of the NPC (namely, the nucleoporins Nup1p and Nup2p, the exportin Cse1p, and the GTPase Gsp1p in its GTP-bound form) accelerate the stepwise disassembly of Kap95pKap60p-NLS-cargo complexes, by triggering allosteric mechanisms in Kap95p and/or Kap60p that cause the rapid release of a bound protein. Therefore, Nup1p, Nup2p, Cse1p, and Gsp1p-GTP function as karyopherin release factors (or KaRFs). We hypothesize that these reactions occur in vivo immediately upon arrival of the Kap95pKap60p-NLS-cargo import complex to the nucleoplasmic side of the NPC, as depicted in Fig. 1.

The Gsp1p-stimulated release of Kap60p from Kap95p via binding to Kap95p (Fig. 5) likely reflects a general mechanism of action of Gsp1p on karyopherin β orthologues dedicated to nuclear import (importin βs). Indeed, Gsp1p-GTP also accelerates the release of Nab2p (an NLS-cargo) from its importin Kap104p (another importin β orthologue) (data not shown).

Thus, Gsp1p likely functions as a generic KaRF for importin βs. In contrast, the Nup2p- and Cse1p-stimulated release of NLS-cargo from Kap60p seems specific for this karyopherin/importin α orthologue. Thus, Nup2p and Cse1p function as karyopherin α-specific KaRFs.

The high affinity of interaction between Kap60p and NLS-cargo in the presence and absence of Kap95p (\(K_D < 149\) pm and \(K_D = 2.8\) nm, respectively), between Kap60p and Kap95p (\(K_D < 153\) pm), and between Nup1pΔN and Kap95pKap60p with or without NLS-cargo (\(K_D = 400\) pm and \(K_D < 51\) pm, respectively) (Fig. 3) predict long interaction times assuming typical rates of molecular association (\(k_{\text{on}} = 10^{-7} \text{M}^{-1} \text{s}^{-1}\)) (32). In fact, the rate of dissociation of Kap60p-NLS-cargo complexes (\(k_{\text{off}} = 1.8 \times 10^{-3} \text{s}^{-1}\)), Kap95p-Kap60p complexes (\(k_{\text{off}} = 3.2 \times 10^{-4} \text{s}^{-1}\)), and Kap60p-Kap60p-NLS complexes (\(k_{\text{off}} = 1.6 \times 10^{-4} \text{s}^{-1}\)) is slow, with half-lives of minutes to hours as expected (Fig. 4 and Table I). Clearly, these reactions occur too slowly on their own to support rapid growth in *S. cerevisiae*, which divide every 2 h. In contrast the Kap95pKap60p-NLS-cargo complex and the Kap95pKap60p heterodimer associate and dissociate very rapidly from Nup1p (Table I) and other FG Nups such as Nup100p and Nup116p (data not shown). Such fast rates of molecular interaction are more compatible with fast transport across the NPC.

The long interaction times between Kap95pKap60p and NLS-cargo (mean interaction time of 104 min) and between NLS-cargo-Kap60p and Kap95p (104 min) are ideal to ensure karyopherin-cargo complex integrity during transport through the NPC. However, they also present a problem, as spontaneous disassembly of the Kap95pKap60p-NLS-cargo complex (Fig. 4, Table I) is too slow as a mechanism for rapid cargo release at the nucleoplasm. Natural selection may have favored the increased stability offered by high affinity interactions while overcoming the disadvantage of slow dissociation by placing “accelerator” molecules at strategic locations within the NPC, such as the nuclear basket structure.

Gsp1p-GTP accelerates the disassembly of Kap95pKap60p-NLS-cargo complexes by increasing by \(\approx 447\)-fold the rate of dissociation of Kap95p from Kap60p-NLS-cargo (from \(k_{\text{off}} = 1.6 \times 10^{-4} \text{s}^{-1}\) to \(7.7 \times 10^{-2} \text{s}^{-1}\)) (Fig. 5 and Table II). Gsp1p-GTP also accelerates disassembly of Kap95pKap60p heterodimers by increasing \(\approx 65\)-fold the rate of dissociation of Kap95p from Kap60p (from \(k_{\text{off}} = 1.6 \times 10^{-4} \text{s}^{-1}\) to \(\approx 2.1 \times\).
10^{-3} \text{s}^{-1} \) (Fig. 5, Table II). Gsp1p-GTP likely concentrates at the nuclear basket structure of the NPC because it binds directly to the nuclear basket nucleoporins Kap60p and Nup2p (14). Also, the Gsp1p-specific guanine exchange factor Prp20p binds directly to Kap60p and Nup2p (14). This implies that Gsp1p-GTP is generated at a site of action in the nuclear basket structure. Altogether these data suggest that Gsp1p-GTP disassembles Kap95p-Kap60p heterodimers (with or without cargo) at the nuclear basket structure of the NPC by triggering an allosteric change in Kap95p structure that causes rapid release of Kap60p. This suggestion is supported by structural studies of similar complexes in crystals, which show partially overlapping binding sites for Ran-GTP (Gsp1p-GTP) and importin α (Kap60p) on importin β (Kap95p) (33).

The Gsp1p-GTP-accelerated release of Kap60p from Kap95p (Fig. 5C) is the reverse of the reported reaction in which Kap60p releases Gsp1p-GTP from Kap95p (34). We speculate that in vivo the equilibrium of this reaction is determined by the cellular compartment in which it takes place. In the cytoplasm, Kap60p is expected to be present in molar excess over free Gsp1p-GTP, thus favoring removal of Gsp1p-GTP from Kap95p and formation of the Kap95p-Kap60p heterodimer. In the nucleus, Gsp1p-GTP is likely more abundant than free Kap60p and may therefore drive the disassembly of the Kap95p-Kap60p heterodimer (Figs. 1 and 5).

Cse1p is the exportin for Kap60p in S. cerevisiae (6, 24, 25). In complex with Gsp1p-GTP, Cse1p also functions as a nuclear import factor because it accelerates disassembly of the Kap60p-NLS-cargo complex by increasing \( k_{\text{off}} \) of NLS-cargo from Kap60p (from \( k_{\text{off}} = 1.8 \times 10^{-3} \text{s}^{-1} \) to \( 6.9 \times 10^{-2} \text{s}^{-1} \)) (Fig. 6 and Table II). Cse1p concentrates at the nuclear basket structure of the NPC (12), and there it may perform its function as a Kap60p-specific KAeF. Our data suggest that Cse1p in complex with Gsp1p-GTP binds Kap60p in Kap60p-NLS-cargo complexes and triggers an allosteric mechanism in Kap60p that causes rapid release of the NLS-cargo (Fig. 6A, diagram). This mechanism may employ the Kap60p intrasteric autoregulatory sequence (IBB domain), which resembles an NLS (31).

Nup2p is localized to the nuclear basket of the NPC (11, 12, 15), binds Kap60p with \( K_D = 0.3 \text{nm} \) (data not shown), and accelerates the disassembly of the NLS-cargo-Kap60p complex (Fig. 7) by increasing more than 22-fold the rate of dissociation of NLS-cargo from Kap60p (from \( k_{\text{off}} = 1.8 \times 10^{-3} \text{s}^{-1} \) to \( 3.9 \times 10^{-2} \text{s}^{-1} \)) (Fig. 7 and Table II). The nuclear basket is an ideal location for Nup2p to encounter and disassemble Kap60p-NLS-cargo complexes that dissociate from Kap95p after Gsp1p-GTP action (see Figs. 1 and 5). Our data suggest that Nup2p binds Kap60p in Kap60p-NLS-cargo complexes, forming a trimeric intermediate that triggers an allosteric change in Kap60p structure, which causes rapid release of the NLS-cargo molecule (Fig. 7). This mechanism may employ the Kap60p intrasteric autoregulatory sequence (34). Because Nup2p is not essential for the survival of S. cerevisiae (35), its observed effect in disassembling Kap60p-NLS-cargo complexes may serve as a backup or replacement for Cse1p-Gsp1p-GTP during times of reduced concentrations of nuclear Gsp1p-GTP. Under such conditions, Nup2p dissociates from Kap60p at the NPC and becomes mobile throughout the cell (14, 15), perhaps to function as a “disaggregase” separating Kap60p from NLS-cargoes. The KAeF activity of Cse1p and Nup2p on Kap60p-NLS-cargo complexes may not be required in cases where the Kap60p-NLS-cargo complex dissociates rapidly on its own.

Nup1p and Nup2p accelerate the disassembly of import complexes by increasing 16–19-fold the rate of dissociation of NLS-cargo from Kap60p-Kap95p heterodimers (from \( k_{\text{off}} = 1.6 \times 10^{-3} \text{s}^{-1} \) to \( 2.5 \times 10^{-3} \text{s}^{-1} \)) (Fig. 8 and Table II). This reaction is orders of magnitude slower than the Nup2p- or Cse1p-Gsp1p-GTP-mediated dissociation of NLS-cargo from Kap60p monomers (Table II), but it is unique in that it uses the Kap60p-Kap95p heterodimer rather than Kap60p monomers as a substrate, and it does not require Gsp1p-GTP action. This mechanism may be most important to yeast when levels of nuclear Gsp1p-GTP are low due to starvation or stress. Because Kap95p and Kap60p bind independently to Nup1pΔN (Fig. 2C) and Nup2p (12, 16), we cannot distinguish whether Nup1pΔN and Nup2p trigger an allosteric change in Kap60p structure, Kap95p structure, or both, that results in release of NLS-cargo from Kap60p. Interestingly, the average time needed for Nup1pΔN to trigger NLS-cargo release from Kap60p-Kap60p (\( t_{\text{avg}} = 276 \text{s} \)) is longer than the average time a Kap95p-Kap60p-NLS-cargo complex spends bound to Nup1pΔN (\( t_{\text{avg}} = 21 \text{s} \)), which implies that a Kap95p-Kap60p-NLS-cargo complex can interact with Nup1pΔN without releasing the NLS-cargo. This explains the presence of NLS-cargo bound to Kap60p-Kap95pNup1pΔN complexes in Fig. 2C.

The dissociation of Kap60p-NLS-cargo complexes from Nup1pΔN (Table I) and other FG Nups is fast. Among all of the Kap-Nup interactions tested the slowest dissociation was the dissociation of Kap95p-Kap60p heterodimers from Nup1pΔN (\( t_{\text{off}} = 21 \text{s} \)) (Table I). Even in that case the dissociation occurs orders of magnitude faster than the slow intrinsic rate of dissociation of NLS-cargo from Kap60p-Kap95p (\( t_{\text{off}} = 73 \text{min} \)), of NLS-cargo from Kap60p (\( t_{\text{off}} = 6–7 \text{min} \)), of Kap60p from
Kap95p ($t_{1/2} = 36$ min), and of Kap95p from Kap60p-NLS-cargo ($t_{1/2} = 73$ min) (Table I). This fast spontaneous dissociation of Kap95p-Kap60p from nucleoporins may occur rapidly enough to support a stochastic nuclear import mechanism. In such a mechanism, karyopherins translocate across the NPC via facilitated diffusion involving a series of fast association and dissociation events with multiple FG Nups (17) (Fig. 1). Possibly, Gsp1p-GTP or additional proteins assist this stochastic mechanism by accelerating the rate of dissociation of karyopherins from nucleoporins. However, our current assay cannot measure dissociation rates on the time scale necessary to test this hypothesis.

The association rate ($k_{on}$) of Kap95p-Kap60p with Nup1p was calculated using the equation $k_{on} = k_{off}/K_D$, the measured affinity of Kap95p-Kap60p toward Nup1pΔN ($K_D \leq 51$ pm), and the measured rate of dissociation of the complex ($k_{off} \approx 3.3 \times 10^{-2}$ s$^{-1}$) (Table I). We find that Kap95p-Kap60p binds Nup1pΔN at a very fast rate ($k_{on} \approx 6.5 \times 10^8$ M$^{-1}$ s$^{-1}$). This rate approaches the limit imposed by molecular diffusion ($\sim 10^8$ M$^{-1}$ s$^{-1}$) (32). We envision that diffusion-limited binding of Kaps to FG Nups may be a fundamental feature of NPC mechanisms. In accordance, the maximal flux of transportin (a Kaps to FG Nups) may occur rapidly enough than the translocation process per se.

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