Molecular Basis for the Rapid Dissociation of Nuclear Localization Signals from Karyopherin α in the Nucleoplasm*

Received for publication, July 9, 2003, and in revised form, September 18, 2003
Published, JBC Papers in Press, September 26, 2003, DOI 10.1074/jbc.M307371200

Daniel Gilchrist and Michael Rexach‡
From the Department of Biological Sciences, Stanford University, Stanford, California 94305-5020

The yeast karyopherin heterodimer Kap60pKap95p facilitates nuclear import of proteins bearing a classic nuclear localization signal (NLS). The α subunit Kap60p binds to the NLS of cargo molecules in the cytoplasm, forming stable complexes that must ultimately dissociate in the nucleoplasm. Although Kap60p can release NLSs on its own using an autoinhibitory sequence (AIS) motif that can occupy the NLS binding site, that mechanism is too slow to support rapid nuclear import. We previously showed that the nuclear basket nucleoporin Nup2p and the exportin complex Cse1pGsp1pGTP function as karyopherin release factors (KaRFs) because they can accelerate the rate of dissociation of NLSs from Kap60p. Here we dissect the molecular mechanics of their KaRF activity. We show that Cse1p accelerates dissociation of Kap60pNLS-cargo complexes and Kap60pNup2p complexes by increasing the affinity of Kap60p for its AIS motif. In contrast, Nup2p uses a conserved sequence motif (VMXXRKIA) coupled to an AIS-like motif to accelerate dissociation of Kap60pNLS complexes in a vectorial reaction mechanism. Mutation of either motif in Nup2p leads to a loss of KaRF activity and to the accumulation of Kap60pNLS-cargo complexes in the nucleoplasm of yeast. We discuss a model whereby Nup2p, Cse1p, and Gsp1p cooperate to establish directionality in the movement of Kap60p and NLS-cargos across the nuclear pore complex.

Transport between the cytoplasm and nucleoplasm of eukaryotic cells occurs at the nuclear membrane and proceeds through nuclear pore complexes (NPCs) (1–5). Proteins that need to be imported into nuclei (cargos) contain nuclear localization signals (NLSs) that are recognized by mobile receptors termed karyopherins (importins/transportins). In Saccharomyces cerevisiae, the karyopherin αβ heterodimer Kap60pKap95p is responsible for import of proteins bearing a cNLS (6). The Kap60p subunit (karyopherin α; Srp1p) binds simultaneously to the NLS-cargo and to the Kap95p subunit (karyopherin β), whereas Kap95p facilitates transport of Kap60pNLS-cargo complexes across the NPC by interacting with nuclear pore complex proteins (nucleoporins; Nups) (7).

A subset of Nups and the Gsp1p GTPase contribute to the efficiency and directionality of Kap95p and Kap60p translocation across the NPC. Nup1p and Nup2p reside in the nuclear basket of the yeast NPC and promote efficient import of Kap95pKap60pNLS-cargo complexes (8–12). Gsp1p (Ran in vertebrates) (13, 14) imparts directionality to their translocation by executing a terminal step in Kap95pKap60p-mediated nuclear import and by serving as a cofactor in the initial step of Kap60p nuclear export. In nuclear import, Gsp1pGTP binds to Kap95p (7) and accelerates release of the Kap60pNLS-cargo complex from Kap95p (15). In nuclear export, Gsp1pGTP binds to the exportin Cse1p and enhances its affinity for Kap60p (its cargo) as the first step in Kap60p export out of the nucleus (16, 17).

Karyopherins bind cargos with high affinity to ensure the integrity of Kap-cargo complexes during transit through the NPC (15, 18–20). Consequently, these interactions may have half-lives of several minutes, as in the case of Kap60pNLS-cargo complexes (15). However, karyopherins need to release cargos rapidly upon completion of translocation to ensure efficient nuclear import. In the case of most importins, binding of Gsp1pGTP disrupts their association with cargos as the terminal step of transport (13, 14, 21). However, in the case of Kap95pKap60pNLS complexes, Gsp1pGTP only accelerates the rate of dissociation of Kap60pNLS from Kap95p, leaving the Kap60pNLS complex intact (15). An essential autoinhibitory sequence (AIS) motif in the importin β binding (IBB) domain of Kap60p (aa 1–61) can occupy the NLS binding pocket of Kap60p and has therefore been proposed to play a key role in the dissociation of Kap60pNLS-cargo complexes (22–24). For some cargos, however, that mechanism is too slow and can take up to several minutes (15). We showed previously that Nup2p and Cse1p function as karyopherin release factors (KaRFs) that accelerate the dissociation of Kap60pNLS-cargo complexes (15). In the case of Cse1p, its KaRF activity was strictly dependent on forming a complex with Gsp1pGTP. Here, we define the molecular basis of Nup2p and Cse1p KaRF activities and explore their relationship to the Kap60p AIS motif.

Experimental Procedures
Preparation and Purification of Recombinant Proteins—Genes encoding the proteins or protein fragments used were amplified from S. cerevisiae genomic DNA using Taq or Pfu-driven polymerase chain reactions with designed oligonucleotides that incorporate restriction enzyme sites compatible for ligation into pGEX-2TK in frame with the 3′ end of the gene encoding glutathione S-transferase (GST). Construction of Kap60p, Kap95p, Cse1p, Gsp1p, Nup2p, and NLS-cargo as GST fusions has been described previously (15). The Kap60AIBB construct lacks aa 1–61. Recombinant proteins were expressed in Escherichia coli strains BLR or BL21 Codon Plus (Novagen) and were purified on glutathione-coated Sepharose beads (Amersham Biosciences). In each case, a bacterial cell extract prepared from 1000 A500 units of cells was incubated with 1 ml of glutathione-coated Sepharose beads in batch for 1 h at 4 °C. After extensive washing of the column, GST proteins were eluted with elution buffer (50 mM Tris, pH 8, 110 mM KCl, 2 mM DTT, dithiothreitol; ONPG, o-nitrophenyl p-D-galactopyranoside).

This paper is available on line at http://www.jbc.org

51937
Mg(OAc)₂, 2 mM DTT. 0.1% Tween 20 plus 15 mM reduced glutathione), and proteins were concentrated in a Centricon 30 unit (Amicon). Concentrated proteins were aliquoted in 1-mg portions, frozen in liquid nitrogen, and stored at −70 °C. To remove GST, thawed GST fusions were treated with thrombin (Calbiochem) to neutralize thrombin, the samples were applied separately to fast protein liquid chromatography Superdex 200 or Superose 6 sizing columns (Amersham Biosciences), which were equilibrated in 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. Tween 20 was 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 (25). Point mutations in Nup2p and Kap60p were incorporated using the QuikChange site-directed mutagenesis kit (Stratagene), and all mutant constructs were verified by DNA sequencing.

RESULTS

Quantitative Yeast Two-hybrid Assay—Yeast expressing Kap60p-GFP were grown to midlog phase at 30 °C in selective media with 2% glucose. 2 μl of culture was spotted on a slide, and yeasts were visualized immediately with a Nikon Eclipse E600 epifluorescence microscope equipped with a CCD camera. Images were captured and adjusted using QED Imaging software and Photoshop 4.0.

We previously showed that Nup2p and Cse1p function as KaRFs (karyopherin release factors) to accelerate the dissociation of Kap60p-NLS-cargo complexes (15). We proposed that their reaction mechanisms proceed through a transient KaRF intermediate where Nup2p or Cse1p-Gsp1p-GTP bind to Kap60p and trigger an allosteric change in its structure that accelerates dissociation of the NLS-cargo. A diagram of these KaRF reaction mechanisms is shown in Fig. 1A (models 1 and 2).

As a starting point in defining the molecular basis of Nup2p and Cse1p KaRF activity, we used a KaRF assay to test if the Kap60p AIS motif in the IBB domain is required by Nup2p or Cse1p-Gsp1p-GTP to accelerate release of Kap60p from NLS-cargo. In a sample KaRF assay (diagrammed in Fig. 1B), the dissociation of radiolabeled Kap60p from NLS-cargo is monitored in the absence (line 1) or presence (line 2) of excess unlabeled Kap60p (control) or in the presence of a possible effector/KaRF (such as Nup2p or the Cse1p-Gsp1p-GTP complex) (line 3). The unlabeled Kap60p serves to prevent rebinding of radiolabeled Kap60p to the NLS-cargo-coated beads during their dissociation reaction, allowing accurate determination of their dissociation rate (15). If the effector is active as a KaRF (line 3), then the dissociation rate of the Kap60p-NLS-cargo complex is accelerated beyond its intrinsic rate.

In all of the KaRF assays shown below, the 5-min data point of control dissociation curves (circled in Fig. 1B, line 2) was normalized to a 0% value, and the dissociation of all radiolabeled Kap60p (circled in Fig. 1B, line 3) was assigned a 100% value. These values are plotted in a histogram format as a qualitative measure of the KaRF activity of possible effectors. In all cases where Gsp1p was used, it was preloaded with GTP; for convenience, however, Gsp1p-GTP is simply referred to as Gsp1p throughout.

To test whether Nup2p and Cse1p-Gsp1p can accelerate release of an NLS-cargo from a Kap60p mutant lacking the IBB domain (Kap60pΔ1IBB; Δaa 1–61) or from a Kap60p point mutant lacking autoinhibitory function (Kap60p K54A) (23), we first had to measure the intrinsic rate of dissociation of these mutant Kap60p-NLS-cargo complexes (Fig. 2, A and B). Radio-
KaRF Reaction Mechanisms

**A. Proposed KaRF Reaction Mechanisms**

1. NLS-cargo + Kap60p → NLS-cargo + Kap60p
2. Kap60p + Nup2p → Kap60p + Nup2p
3. Kap60p + Cse1p + Gsp1p → Kap60p + Cse1p + Gsp1p

**B. A Sample KaRF Assay**

1. Buffer
2. Unlabeled Kap60p (control)
3. Nup2p or Cse1p-Gsp1p (effector/KaRF)

**Fig. 1. Proposed KaRF reaction mechanisms and sample KaRF assay.** A. Diagrams depicting proposed KaRF reaction mechanisms. Model i, Nup2p accelerates dissociation of Kap60p-NLS-cargo complexes by forming a transient KaRF intermediate with Kap60p (depicted in brackets), which triggers an allosteric change in Kap60p structure that destabilizes its interaction with NLS-cargo. Model ii, Cse1p-Gsp1p accelerates dissociation of Kap60p-Nup2p complexes by forming a transient KaRF intermediate, which triggers an allosteric change in Kap60p structure that destabilizes its interaction with Nup2p. Model iii, Cse1p-Gsp1p accelerates dissociation of Kap60p-NLS-cargo complexes by forming a transient KaRF intermediate with Kap60p, which triggers an allosteric change in the Kap60p structure that destabilizes its interaction with NLS-cargo. The gray tether symbols mark the immobilized protein used in KaRF assays, and the radioactivity symbol marks the radiolabeled protein used. Brackets indicate transient KaRF intermediates. B, a sample KaRF assay. The dissociation of radiolabeled Kap60p from beads containing immobilized NLS-cargo is monitored in the presence of buffer alone (line 1) or buffer with unlabeled Kap60p (line 2) (control) or buffer with an effector protein (a KaRF) (line 3). The unlabeled Kap60p is added in molar

**excess to prevent rebinding of radiolabeled Kap60p to beads (line 2); this allows accurate determination of the dissociation rate (control). If the dissociation rate is faster when an effector is added instead of unlabeled Kap60p, then the effector is considered to be a KaRF. For qualitative comparisons, KaRF activity can be scored at \( t = 5 \) min and plotted with values ranging from 0% (the amount of Kap60p released after 5 min in the control) to 100% (all radiolabeled Kap60p released from the immobilized NLS-cargo).**
Fig. 2. Cse1p and Nup2p use distinct mechanisms to accelerate the dissociation of Kap60p-NLS-cargo complexes. A and B, dissociation rate of Kap60pΔIBB-NLS-cargo and Kap60p K54A-NLS-cargo complexes. Beads containing immobilized GST-NLS-cargo (0.3 μg in 60 μl of beads, or 80 nM within beads) were incubated for 2 h at 4 °C with radiolabeled Kap60pΔIBB or Kap60p K54A (0.13 μg added per 60 μl of beads, for a maximum of 40 nM bound within beads) before being diluted 100 times into room temperature buffer containing no additions (diamonds) or...
Cse1p-Gsp1p Enhances the Affinity of Kap60p for Its IBB Domain—How does Cse1p-Gsp1p employ the Kap60p IBB domain to accelerate the release of NLS-cargo from Kap60p? In one scenario, Cse1p-Gsp1p might trigger an allosteric change in Kap60p structure that reduces its affinity toward the NLS-cargo, allowing the IBB domain to compete more effectively for the NLS-binding site. That scenario is unlikely, since Cse1p-Gsp1p cannot accelerate dissociation of NLS-cargo from Kap60pΔIBB (Fig. 2C). In a second scenario, Cse1p-Gsp1p might accelerate release of an NLS from Kap60p by increasing the affinity of Kap60p toward the AIS motif in its IBB domain. To test this, we examined the effect of Cse1p-Gsp1p on Kap60p binding to an isolated IBB domain in a solution binding assay (Fig. 2E). The IBB domain of Kap60p was immobilized on beads as a GST fusion (GST-IBB) and was incubated with Kap60p or Kap60pΔIBB in the presence or absence of Cse1p-Gsp1p for 1 h at 4 °C. Neither Kap60p nor Kap60pΔIBB bound to the isolated IBB domain (Fig. 2E, lanes 2 and 4), suggesting that Kap60p is normally in a conformation that exhibits low affinity toward the IBB domain. However, in the presence of Cse1p-Gsp1p, Kap60pΔIBB bound stably to the immobilized IBB domain and formed a complex with Cse1p-Gsp1p (lane 5). In contrast, Cse1p-Gsp1p did not promote binding of full-length Kap60p to the immobilized IBB domain (lane 3), suggesting that wild type Kap60p binds its IBB domain in cis more effectively than in trans. In a similar experiment, Nup2p did not stimulate binding of Kap60p or Kap60pΔIBB to the immobilized IBB domain (data not shown). Altogether, the data suggest that Kap60p is normally in a conformation that binds weakly to its IBB domain, but Cse1p-Gsp1p binding can trigger an allosteric change in Kap60p structure that significantly enhances its affinity for the IBB domain. This allosteric conformation switch may be the molecular basis for Cse1p-Gsp1p KaRF activity (i.e. changing the Kap60p NLS-cargo binding pocket from a pro-NLS to a pro-IBB conformation).

Cse1p Also Accelerates Dissociation of Kap60p from Nup2p via a KaRF Mechanism—It has been previously shown that Cse1p-Gsp1p recognizes the interaction of Kap60p with Nup2p, and it was proposed that Nup2p serves as a platform for Cse1p-mediated export of Kap60p from the nucleus (10, 11, 28). We therefore examined whether Cse1p-Gsp1p can also accelerate the rate of dissociation of Kap60p from Nup2p.

To test this notion, we first had to measure the dissociation rate of radiolaabeled Kap60p from immobilized Nup2p (Fig. 3A). At room temperature, this complex had a very short half-life (t_1/2 = 3 × 10⁻² s⁻¹) (data not shown) despite its high affinity (K_D = 0.3 nM), making the analysis difficult. To solve that problem, we monitored the same reaction at low temperature, which slows down the dissociation rate. At 4 °C, the Kap60p-Nup2p interaction had a half-life of 4.5 min in the presence of excess unlabeled competitor Kap60p (k_off = 2.5 × 10⁻⁶ s⁻¹) (Fig. 3A). In comparison, the Kap60pΔIBB-Nup2p complex dissociated more slowly (data not shown).

The dissociation of Kap60p and Kap60pΔIBB from Nup2p was monitored in a KaRF assay in the presence of Cse1p-Gsp1p or Nup2p as possible effectors (Fig. 3B). Cse1p-Gsp1p accelerated the dissociation of Kap60p but not Kap60pΔIBB from Nup2p (Fig. 3B), consistent with a KaRF mechanism whereby Cse1p-Gsp1p binds to Kap60p (in a Kap60p-Nup2p complex) and triggers an allosteric change in Kap60p structure that accelerates the release of Nup2p in an IBB-dependent mechanism (Fig. 1A, model ii). A stable KaRF intermediate containing Nup2p-Kap60pΔIBB-Cse1p-Gsp1p was captured using a solution binding assay (Fig. 3C). Beads coated with Nup2p containing were incubated with Kap60p or Kap60pΔIBB for 30 min, washed once, and then incubated for 1 h in the presence or absence of Cse1p and Gsp1p-GTP. In the presence of Cse1p-Gsp1p, nearly all Kap60p was recovered in the unbound fraction (lane 3), consistent with the KaRF activity of Cse1p-Gsp1p. In contrast, the Kap60pΔIBB mutant protein remained bound to Nup2p in the presence of Cse1p-Gsp1p, and a fraction of the Cse1p-Gsp1p added was recovered in the bound fraction in a stable complex with Nup2p-Kap60pΔIBB (lane 5).

The IBB Domain of Kap60p Controls the Directionality of the Nup2p KaRF Reaction—The Nup2p-mediated release of NLS-cargo from Kap60p could impart directionality to the Kap60p-Kap95p-mediated nuclear import pathway if it was an irreversible last step of transport. To test this notion, we examined whether NLS-cargo could accelerate release of Kap60p from Nup2p in a KaRF assay (i.e. we tested the reverse reaction of the one shown in Fig. 1A, model i). The presence of 200 nM NLS-cargo (or even 800 nM NLS-cargo) had no effect on the disassembly of Kap60p-Nup2p complexes (Fig. 3D, black bars), demonstrating that an NLS-cargo cannot accelerate release of Kap60p from Nup2p. Surprisingly, however, 200 nM NLS-cargo did accelerate the release of the Kap60pΔIBB mutant from immobilized Nup2p (gray bars), despite the fact that Kap60pΔIBB dissociates more slowly from Nup2p than full-length Kap60p (data not shown). Thus, the Nup2p KaRF mechanism can be an irreversible last step in transport, but only when the Kap60p IBB domain is available for binding Kap60p.

A Conserved Sequence Motif in a Compact Domain of Nup2p That Binds Kap60p—To define the Nup2p KaRF mechanism in more detail, we searched for amino acid sequences within Nup2p that bind Kap60p. As a first clue, we noted that Nup1p and Nup2p are unique among yeast FG Nups in that they bind Kap60p monomers directly (10, 11, 28, 29) (see Fig. 5A for an example). Also, a Kap60p binding site on Nup1p was previously mapped to the C terminus of Nup1p (28, 29) and to the
N-terminal 172 amino acids of Nup2p (10, 28). A comparison of the two regions revealed a sequence motif, VMXXRKIA, which is highly conserved between Nup1p and Nup2p (Fig. 4A) and their orthologs in other budding yeast (data not shown). Immediately downstream of the conserved VMXXRKIA motif, the Nup2p orthologs (but not the Nup1p orthologs) contain the amino acid sequence KRR (Fig. 4A; data not shown). Interestingly, that sequence is a key element of the AIS motif in the Kap60p IBB domain (22, 23).

To test whether the VMXXRKIA sequence motif is part of a Kap60p binding site in Nup2p and Nup1p, we performed solution binding assays using small fragments of Nup2p and Nup1p that contain the conserved motif. Nup2p aa 26–51 and Nup1p aa 1059–1076 were immobilized on beads as GST fusion proteins. The results are shown in Fig. 4B. The binding of Kap60p to GST-Nup2p and GST-Nup1p was determined by SDS-PAGE and Coomassie Blue staining. The asterisks mark fragments of GST-Nup2p and Cse1p.
sions and incubated with purified Kap60p. Kap60p bound to Nup2p aa 26–51 (Fig. 4B, lane 1) and to Nup1p aa 1059–1079 (data not shown) in a reaction that was effectively competed by a soluble Nup1p peptide containing the conserved sequence motif (Nup1p aa 1059–1076) (lane 3). Because the compact domains of Nup2p and Nup1p that bind Kap60p exhibit a loose resemblance to classic NLSs (in that they contain a cluster of positively charged amino acids) (Fig. 4A), we also tested whether a cNLS-peptide could compete for binding. The cNLS peptide used contains the SV40 T-antigen monopartite NLS, which is an efficient competitor of Kap60p binding to cNLS-containing proteins (data not shown). The cNLS-peptide did not compete for binding of Kap60p to Nup2p (Fig. 4B, lane 2) or Nup1p (data not shown); instead, it enhanced the binding. From these experiments, we conclude that Kap60p binds to the compact 26-amino acid domain of Nup2p (which contains the VMXXRKIAAKKRRR motif) in a manner that is different from its binding to a classical monopartite NLS.

The enhanced binding of Kap60p to the 26-aa fragment of Nup2p in the presence of a cNLS peptide implies that Kap60p can bind simultaneously to full-length Nup2p and the NLS of a cargo molecule. The enhanced binding also implies that the NLS can increase the affinity of Kap60p for Nup2p. To test the first notion, we examined the binding of Kap60p to an immobilized NLS-cargo (containing the NLS of Cbp80p) in the presence and absence of Nup2p (Fig. 4C). Despite the fact that Nup2p antagonizes the binding of Kap60p to the NLS-cargo (as expected from its KaRF activity), a small portion of Nup2p was captured in a stable complex with the NLS-cargo and Kap60p (top panel, lane 2), providing evidence of a KaRF intermediate as depicted in Fig. 1A (model i). We did not test the effect of NLS-cargo on the affinity of Kap60p to Nup2p, because that affinity ($K_D = 0.3$ nM) already borders the detection limit of our affinity assay.

**Fig. 4.** Nup2p contains a conserved Kap60p binding site coupled to an ALS-like motif. A, amino acid sequence homology between the Kap60p binding regions of Nup1p and Nup2p reveals a conserved sequence motif. Plus signs mark basic amino acids, and dots represent conservative changes in the amino acid sequences. B, Kap60p binds to a compact domain of Nup2p that contains the conserved sequence motif. Immobilized GST-Nup2p aa 26–51 (2 μg in 10 μl of beads or 7.6 μM within beads) was incubated for 1 h at 4 °C with Kap60p (0.5 μg added per 10 μl of beads, or 0.8 μM) alone or in combination with a 300-fold molar excess of a peptide that mimics the NLS of the SV40 large T-antigen (NLS peptide) or a peptide that mimics the last 18 amino acids of Nup1p (Nup1p aa 1059–1076) (6 μg of peptide added per 10 μl of beads). C, Kap60p can bind simultaneously to Nup2p and an NLS-cargo. Immobilized GST-NLS-cargo (2 μg in 10 μl of beads or 3.3 μM within beads) was incubated for 1 h at 4 °C with Kap60p (1 μg per 10 μl of beads, or 1.2 μM). Beads were washed twice and incubated for 1 h at 4 °C with or without Nup2p (1 μg added per 10 μl of beads, or 1.6 μM). Bound and unbound proteins were collected, resolved by SDS-PAGE, and stained with Coomassie Blue. The asterisks mark fragments of Kap60p and Nup2p.

Mutations in Nup2p That Disrupt Its KaRF Activity toward Kap60p-NLS-cargo Complexes—Point mutagenesis was used to determine which of the conserved amino acids in the Nup2p VMXXRKIAAKKRRR sequence motif might be important for its KaRF activity. Three Nup2p mutants were generated (R39A, I41A, and 43KRR → AAA) and tested for their ability to bind Kap60p (Fig. 5A) and their ability to accelerate release of radiolabeled Kap60p from immobilized NLS-cargo (Fig. 5B). None of the mutations affected binding of Nup2p to Kap60p (Fig. 5A, lane 3; data not shown), but the Nup2p I41A and KRR → AAA mutants displayed a lack of KaRF activity relative to wild type Nup2p (Fig. 5B). The Nup2p R39A mutant had no KaRF defect (data not shown). These data demonstrate that the Nup2p I41A and Nup2p KRR → AAA mutants are inactive as KaRFs, despite the fact that they bind Kap60p as well as wild type Nup2p.

We also examined whether the Nup2p mutants could bind Kap95p monomers and Kap95p-Kap60p heterodimers. Nup2p
Fig. 5. Mutations in Nup2p disrupt its KaRF activity without affecting its ability to bind Kap60p. A, binding of Kap60p and Kap95p to wild type and mutant Nup2p. GST-Nup2p or GST-Nup2p mutants were immobilized on beads (1.5 μg in 10 μl of beads or 1.5 μm within beads) and mixed with Kap60p and Kap95p (1 μg of each per 10 μl of beads or 1.6 and 1 μm, respectively) as indicated. After 1 h at 4°C, bound and unbound proteins were collected as before, resolved by SDS-PAGE, and stained with Coomassie Blue. The asterisks mark a fragment of GST-Nup2p. B, Nup2p I41A and Nup2p KRR → AAA mutations lack KaRF activity. Beads containing immobilized GST-NLS-cargo (0.3 μg in 60 μl of beads or 80 nm within beads) were incubated for 2 h at 4°C with radiolabeled Kap60p (0.13 μg added per 60 μl of beads for a maximum of 40 nm within beads) before being diluted 100 times into buffer containing 200 mM unlabeled competitor Kap60p (Control) or 200 mM Nup2p, Nup2p I41A, or Nup2p KRR → AAA, as possible effectors. The samples were processed as described in the legend to Fig. 2A.

I41A and Nup2p KRR → AAA bound to Kap95p-Kap60p heterodimers just as well as wild type Nup2p (Fig. 5A, lane 3), but the Nup2p KRR → AAA mutant was surprisingly defective in binding Kap95p monomers (lane 2, bottom panels). This unexpected result implies that Kap95p monomers recognize the KRR sequence of Nup2p, possibly as an NLS.

In Vivo Effects of Impaired Nup2p and Cse1p KaRF Activity—If Nup2p and Cse1p function in vivo to accelerate the dissociation rate of NLS-cargos from Kap60p in the nucleus, then elimination of Nup2p and/or Cse1p KaRF activity should result in increased persistence of Kap60p-NLS-cargo complexes in the yeast nucleoplasm. To test this, we used a yeast two-hybrid assay that uses β-galactosidase as a reporter to quantify the persistence of nuclear Kap60p-NLS-cargo complexes. The two-hybrid assay is perfectly suited for this purpose, because it exclusively detects protein interactions that occur within the nucleoplasm of yeast and because the levels of β-galactosidase expression can reflect the abundance and persistence of the protein interaction being tested.

Two-hybrid plasmids were constructed that encode (i) the SV40 T-antigen NLS coupled to the Gal4p activation domain (cNLSAD) and (ii) Kap60p or the Kap60p mutants Kap60p ED and Kap60p K54A fused to the Gal4p DNA binding domain (Kap60pDBD, Kap60p EDDBD, and Kap60p K54A DBD). The Kap60p ED mutant is useful as a negative control, because it cannot bind cNLSs (22, 30). The Kap60p K54A mutant is defective in Cse1p-mediated dissociation of Kap60p-NLS-cargo complexes (Fig. 2B) and autoinhibition of NLS binding (23) and is therefore useful to test the in vivo role of Cse1p KaRF activity and Kap60p autoinhibition. The two-hybrid plasmid pairs were introduced into a yeast strain (Y187) that harbors a chromosomal β-galactosidase reporter gene under control of the GAL4 promoter. Finally, to also test the role of Nup2p KaRF activity, we deleted the NUP2 gene (nup2Δ) in that strain and reintroduced it as wild type NUP2 or mutant nup2 I41A or nup2 KRR → AAA, all under the control of the wild type NUP2 promoter using plasmid pRS313.

Yeast (Y187, nup2Δ) carrying the two-hybrid plasmids and the Nup2p-expressing plasmids were grown on synthetic medium to midlog phase, and the β-galactosidase activity in cell extracts was quantified by measuring the hydrolysis rate of the chromogenic β-galactosidase substrate ONPG. Importantly, the Nup2p I41A and Nup2p KRR → AAA proteins were expressed to the same level as wild type Nup2p, as judged by Western blot analysis of whole cell extracts (Fig. 6A). Also, the cellular location of Nup2p mutants was the same as wild type, showing punctate nuclear rim staining by indirect immunofluorescence (Fig. 6B). All of the strains showed comparable expression of cNLSAD and Kap60p DBD fusion proteins (Fig. 6C, bottom panels). Results using the nup2Δ parent strain were not included, because the cNLSAD chimera is not expressed in nup2Δ knockout cells (nup2Δ) unless wild type Nup2p or Nup2p mutants are expressed from a plasmid.

First, we find that yeast expressing wild type Nup2p and the cNLSAD and Kap60p DBD chimeras contained very low (but detectable) levels of β-galactosidase activity (0.15 ± 0.05 A415/min) (Fig. 6C, top panel, lane 1). This suggests that Kap60p DBD and cNLSAD can form short lived complexes in the yeast nucleus. This small amount of β-galactosidase activity reflected the interaction between Kap60p DBD and the cNLSAD chimeras, because the strain carrying the mutant form of Kap60p that cannot bind NLSs (Kap60p DBD ED) displays no β-galactosidase activity above background (Fig. 6C, top panel, lane 3). To test the role of Cse1p KaRF activity in vivo, we complemented using the mutant cse1-I-1 strain background. However, because the cse1-I protein (D220N) is impaired in binding Kap60p (data not shown), it was not useful to address Cse1p KaRF activity directly. Instead, we used the K54A mutant of Kap60p, which binds normally to Cse1p (in the presence of
Gsp1p-GTP) but is unresponsive to Cse1p KaRF activity (Fig. 2B). A caveat is that the Kap60p K54A mutant is also defective in autoinhibition of NLS binding (23). Wild type cells (NUP2) expressing Kap60p K54A DBD and the cNLS AD chimera showed increased β-galactosidase activity relative to cells expressing wild type Kap60p DBD or the control Kap60p ED DBD (Fig. 6C, top panel, compare lane 2 with lanes 1 and 3). This result suggests that disruption of the Kap60p AIS motif (via the K54A mutation) causes Kap60p NLS interactions to persist in the nucleoplasm of yeast. This could result from the inability of Kap60p to undergo autoinhibition of NLS binding (as stated above) or from the inability of Cse1p to use the Kap60p AIS motif to accelerate dissociation of Kap60p NLS complexes or both. Alternatively, the increased β-galactosidase activity could reflect the fact that Kap60p K54A accumulates in the nucleoplasm of cells to higher levels than wild type Kap60p (23).

We then examined the effect of Nup2p KaRF mutants on the nuclear persistence of Kap60p NLS-cargo complexes (Fig. 6C, lanes 4–9). Nup2p I41A and Nup2p KRR → AAA yeast expressing the wild type Kap60p DBD and the cNLS AD chimeras (lanes 4 and 7) showed a large increase in β-galactosidase activity relative to wild type NUP2 yeast carrying the same chimeras (lane 1). This reflects an accumulation of Kap60p NLS-cargo complexes in the yeast nucleoplasm when the KaRF activity of Nup2p is compromised by a mutation in its VMXRRK1-
AXXKRR motif. Notably, nup2 I41A cells showed a 2-fold greater β-galactosidase activity than nup2 KRR → AAA cells (compare lanes 4 and 7), implying that the I41A mutation in Nup2p results in a more severe KaRF defect than the KRR → AAA mutation.

Finally, we examined the combined effect of disrupting all three known mechanisms of NLS-cargo release from Kap60p: namely the Kap60p autoinhibitory mechanism, the Cse1p-mediated mechanism, and the Nup2p mechanism. nup2 I41A and nup2 KRR → AAA mutant cells expressing the Kap60p K54AΔAD and the cNLSΔAD chimeras showed the greatest β-galactosidase activity of all strains examined (Fig. 6C, lanes 5 and 8). It is interesting that the β-galactosidase activity in these triply deficient cells is greater than the sum of β-galactosidase activity from cells individually disrupted in Nup2p (lanes 4 and 7) or Kap60p autoinhibition and Cse1p KaRF activity (lane 2). This suggests that all three mechanisms of NLS-cargo release from Kap60p act synergistically to promote efficient dissociation of Kap60p-NLS-cargo complexes in the yeast nucleoplasm.

Kap60p Localization in Nup2p KaRF Mutant Cells—We have shown that loss of Nup2p KaRF activity results in persistence of Kap60p-NLS-cargo complexes in the yeast nucleoplasm (Fig. 6C). This may indirectly cause a decreased efficiency of NLS-cargo import by delaying the export of Kap60p from the nucleus. To test this notion, we examined the localization of Kap60p in cells with impaired Nup2p KaRF activity (Fig. 7A). Yeasts lacking Nup2p (nup2Δ) and expressing chromosomally tagged Kap60p-GFP were transformed with a centromeric plasmid containing either wild type NUP2, nup2 I41A, or nup2 KRR → AAA alleles under the wild type NUP2 promoter. Kap60p-GFP was then visualized with live cell fluorescence microscopy. In Nup2p and nup2 KRR → AAA mutant yeast, Kap60p-GFP was evenly distributed throughout the cytoplasm and nucleoplasm of cells, with some concentration at the nuclear rim (Fig. 7A, a and c). In contrast, nup2Δ and nup2 I41A yeast showed increased nuclear accumulation of Kap60p-GFP (Fig. 7A, b and d).

Rate of Kap60p-mediated Nuclear Import in Nup2p KaRF Mutant Cells—We employed an assay that allows quantitation of the nuclear import rate of NLS-cargo in vivo (32) to test whether loss of Nup2p KaRF activity causes a decreased efficiency of nuclear import of NLS-cargos (Fig. 7B). nup2Δ yeast expressing cNLS-YFP (a Kap60p-dependent cargo) was transformed with centromeric plasmids containing wild type NUP2, nup2 I41A, or nup2 KRR → AAA alleles under the wild type NUP2 promoter. All of the strains showed similar steady-state nuclear accumulation of cNLS-YFP (not shown). When cells were poisoned with azide and 2-deoxyglucose to deplete cellular ATP and GTP, cNLS-YFP equilibrated equally between the nucleus and cytoplasm due to its small size (29 kDa). Following the removal of poison, the time-dependent nuclear reaccumulation of cNLS-YFP was quantified (Fig. 7B). After 5 min, 85% of wild type NUP2 yeast showed strong nuclear accumulation of the reporter cNLS-YFP (open circles), whereas only 50% of nup2Δ and nup2 I41A yeast displayed nuclear accumulation of cNLS-YFP within 5 min (closed circles and open squares, respectively).
KaRF Reaction Mechanisms

KaRF Reaction Mechanisms

In the simplest mechanism, Kap60p uses an essential autoinhibitory sequence (the AIS motif) in its N-terminus (terminus (the IBB domain) to disrupt importin-cargo complexes as they enter the nucleoplasm; this includes all karyopherin subfamilies. However, in the case of the essential Kap60p-Kap95p heterodimer, Gsp1p-GTP only causes dissociation of the Kap60p-NLS-cargo complex from Kap95p, leaving a stable Kap60p-NLS-cargo complex that can persist for minutes (15). Failure to dissociate Kap60p-NLS complexes in vivo can lead to the persistence of Kap60p-NLS-cargo complexes in the nucleoplasm (Fig. 6C), defects in Kap60p export (Fig. 7A) (23), defects in Kap60p-mediated nuclear import (Fig. 7B), and even cell death as in the case of the Kap60p K54A mutation (23). Moreover, dissociation of Kap60p-NLS-cargo complexes is crucial in the timing of cell cycle events, such as dissociation of TPX2 from karyopherin/importin α (30, 33). Thus, to ensure that Kap60p-NLS complexes do not persist in the nucleoplasm, yeasts use three mechanisms to accelerate the dissociation of NLSs from Kap60p.

Three Distinct Mechanisms Accelerate the Dissociation of NLSs from Kap60p—In the simplest mechanism, Kap60p uses an essential autoinhibitory sequence (the AIS motif) in its N-terminus (the IBB domain) to disrupt autoinhibition (the AIS motif, KRR) (24). We therefore propose the stability of the interaction (data not shown). Altogether, the data are consistent with a mechanism whereby Nup2p binds to the Kap60p-NLS-cargo complex and triggers an allosteric change in Kap60p structure that increases the dissociation rate of the NLS (Fig. 1A, model i). It is interesting that the KRR sequence in the Nup2p KaRF domain mimics the key sequence determinant in the Kap60p IBB domain responsible for autoinhibition (the AIS motif, KRR) (24). We therefore speculate that Nup2p accelerates release of an NLS from Kap60p using a molecular mechanism that is (in essence) similar to the autoinhibitory mechanism of Kap60p (i.e. displacement of the NLS by Nup2p via partially overlapping binding sites in the concave groove of Kap60p).

The observation that an NLS peptide stimulates binding of Kap60p to Nup2p aa 26–51 (Fig. 4B) seems inconsistent with the fact that full-length Nup2p stimulates dissociation of NLSs from Kap60p (Figs. 2C and 4C) (15). However, if one assumes that Nup2p KaRF activity requires residues outside of this small domain of Nup2p (aa 26–51), it would explain why the complex between NLS peptide, Kap60p, and Nup2p aa 26–51 appears to be stable and perhaps even reflective of a KaRF intermediate (Fig. 1A, model i). The fact that an NLS peptide stimulates binding of Kap60p to Nup2p aa 26–51 (Fig. 4B) also implies that Kap60p has a higher affinity for Nup2p (i.e. a slower off-rate) when it is in complex with an NLS. In essence, it may be easier for Nup2p to bind Kap60p when it has an NLS rather than the autoinhibitory sequence of the IBB domain occupying its concave groove. From the point of view of Nup2p efficiency as a KaRF in nuclear import, it would make sense that Nup2p has a preference for interaction with NLS-occupied Kap60p rather than “empty” or “autoinhibited” Kap60p.

Modulation of Nup2p KaRF Activity—The cellular location of Nup2p may affect its KaRF activity. Nup2p is normally tethered to the nuclear basket of the yeast NPC via Nup60p, but it becomes mobile (i.e. shuttles between the nucleoplasm and cytoplasm) when the cellular concentration of Gsp1p-GTP is low (9, 26). While bound to the nuclear basket of the NPC, however, it is likely that Nup2p is active as a KaRF. For
instance, a Nup2p mutant (Nup2p Δaa1–50) that cannot bind Kap60p (and therefore lacks KaRF activity) binds normally to Kap60p and their auxiliary "bridging factor" Gsp1p-GTP (9, 26). This implies that the KaRF domain of Nup2p and the Kap60p binding domain are physically separable, consistent with the notion that the Nup2p KaRF domain is exposed while Nup2p is bound to Kap60p. However, under conditions of low Gsp1p-GTP, Nup2p forms complexes with Kap95p monomers and Kap95p–Kap60p heterodimers, which cause its dissociation from Kap60p (9, 26) and probably facilitate its nucleocytoplasmic shuttling (36). As discussed below, the KaRF activity of Nup2p is inhibited by Kap95p where the concentration of free Gsp1p-GTP is low. We therefore speculate that Nup2p is active as a KaRF in the nucleoplasm and at the nuclear basket structure, where the concentration of Gsp1p-GTP is low and allows complex formation with (its inhibitor) Kap95p.

Evidence indicates that Kap95p functions as an inhibitor of Kap60p-NLS-cargo dissociation. First, sequestration of the Kap60p IBB domain (via binding to Kap95p) may prevent autoinhibition of Kap60p; this is consistent with the >18-fold increase in the affinity of Kap60p for an NLS in the presence of Kap95p (15). Second, sequestration of the Kap60p IBB domain by Kap95p probably prevents the Cse1p-mediated dissociation of Kap60p-NLS-cargo complexes, because Cse1p requires the Kap60p IBB domain to function as a KaRF (Fig. 2B). Third, Nup2p has a lower KaRF activity toward Kap95p–Kap60p-NLS-cargo complexes as compared with Kap60p-NLS-cargo complexes (15). Finally, sequestration of Kap60p IBB domain by Kap95p probably compromises the directionality of the Nup2p-mediated dissociation of Kap60p-NLS-cargo complexes, as happens in the absence of the IBB domain (Fig. 3D). In conclusion, we suggest that Kap95p binding to Kap60p and/or Nup2p acts as an inhibitor to prevent release of NLS-cargos from Kap60p throughout the cell, except in places where the concentration of Gsp1p-GTP is sufficiently high to disrupt the association of Kap95p with Kap60p and/or Nup2, such as in the nucleoplasm or the nuclear basket of the NPC.

It is interesting that the Nup2p KRR → AAA and I14A mutants are defective in KaRF activity in vitro (Fig. 5B) and in vivo (Fig. 6C, lane 7), but only the I14A mutant affects Kap60p cellular localization and the rate of cNLS nuclear import in vivo (Fig. 7, A and B). This unexpected result can be explained by the fact that the I14A mutation is more disruptive to Nup2p KaRF activity than the KRR → AAA mutation. This notion is supported by the observation that Kap60p-NLS-cargo complexes persist longer in the nucleoplasm of nup2 I14A yeast in comparison with nup2 KRR → AAA yeast (as reflected by the 2-fold greater production of β-galactosidase in the nup2 I14A mutant) (Fig. 6C, compare lanes 4 and 7). It follows that the Nup2p KRR → AAA mutant protein may not be sufficiently defective to slow down the Kap60p-mediated nuclear import pathway or to cause nuclear accumulation of Kap60p. Another key difference between the I14A and KRR → AAA mutation is their effect on Kap95p binding to Nup2p, as judged from the decreased amount of Kap95p monomer bound to Nup2p KRR → AAA (Fig. 5A, lane 2). Indeed, the KRR motif in Nup2p appears to form part of a binding site for Kap95p monomers, whereas residue Ile41 in Nup2p does not. Given that the KRR motif contributes positively to the KaRF activity of Nup2p as well as negatively (by attracting Kap95p as an inhibitor, as discussed above), the KRR mutation could abrogate both contributions (in essence canceling each other out), leaving the rate of cNLS nuclear import and the steady-state localization of Kap60p unaffected. This is different from the I14A mutation, which reduces the ability of Nup2p to dissociate Kap60p-NLS-cargo complexes but retains the ability to bind Kap95p as an inhibitor.

*Nup2p Executes an Irreversible Last Step in Nuclear Import*—The Nup2p-mediated release of NLS from Kap60p appears to be an irreversible, final step in nuclear protein import. Indeed, Nup2p can accelerate the release of an NLS-cargo from Kap60p (Fig. 2A), but the NLS-cargo cannot accelerate release of Nup2p from Kap60p (Fig. 3D). This vectorial reaction mechanism may impart directionality to the Kap60p-dependent nuclear import pathway in vivo without relying on the function of Gsp1p-GTP, which is currently thought to be responsible for imparting all directionality to nucleocytoplasmic transport. In fact, contrary to that notion, the accelerated dissociation of the Kap95p-Kap60p complex by Gsp1p-GTP (15) is fully reversible, since Kap60p monomers can accelerate the dissociation of Kap95p-Gsp1p-GTP complexes (29). In vivo, sequestration and nuclear export of Kap60p by Nup2p and Cse1p–Gsp1p-GTP after Gsp1p dissociates Kap95p from Kap60p may play a key step in establishing the vectoriality of nuclear import via Kap95p–Kap60p heterodimers. It is interesting that the absence of the Kap60p IBB domain has no effect on the Nup2p KaRF mechanism (Fig. 2C) yet renders this otherwise irreversible step fully reversible (Fig. 3D). This suggests that the IBB domain of Kap60p blocks the NLS-binding site (of Kap60p) after Nup2p accelerates dissociation of the NLS-cargo. This finding underscores the importance of the AIS motif in the Kap60p IBB domain in all three mechanisms of Kap60p-NLS-cargo dissociation.

In vivo, Nup2p may play a larger role than Cse1p in dissociating Kap60p-NLS complexes. This speculation is based on the observation that cells deficient in Nup2p KaRF activity (nup2 I14A and nup2 KRR → AAA) show a 2–3-fold greater persistence of Kap60p-NLS complexes in their nucleoplasm compared with cells carrying only a disrupted Kap60p AIS motif (Fig. 6C, compare lanes 4 and 7 with lane 2), which functionally abrogates the autoinhibitory mechanism of Kap60p (23) and the Cse1p-dependent KaRF mechanism (Fig. 2C). Despite its apparent leading role, the Nup2p-dependent release of NLSs from Kap60p is not an essential mechanism, because yeasts survive without Nup2p (31).

*Conservation of Nup2p KaRF Mechanism during Evolution*—The Nup2p KaRF mechanism may be evolutionarily conserved, such that the vertebrate homolog Nup50/Npap60 (37) may also function to accelerate dissociation of karyopherin α-NLS-cargo complexes in the nucleoplasm. This notion is consistent with the demonstration that vertebrate karyopherin α/importin α binds simultaneously to Nup50 and an NLS-cargo (37). This is similar to Nup2p, which can bind a Kap60p-NLS complex as part of a KaRF intermediate (Fig. 4C). Interestingly, the human Nup50 contains the sequence WXXRAIKKXXR40 at its N terminus, which resembles a component of the KaRF domain in the N terminus of Kap60p (VMXXKIAAXXXKRR). Last, Nup50 and Nup2p are both dynamic constituents of the nuclear basket structure that can detach to shuttle between the nucleoplasm and the cytoplasm (37). Regardless, the functional similarity between Nup50 and Nup2p will need to be addressed directly by testing the KaRF activity of Nup50 on karyopherin α-NLS-cargo complexes.

The yeast nucleoporin Nup1p also contains a VMXXKIA sequence motif at its C terminus (Fig. 4A). This sequence forms part of a binding site (Fig. 4B and data not shown) but lacks the coupled AIS-like KRR motif present in Nup2p. This may explain why Nup1p binds Kap60p but does not exhibit KaRF activity toward Kap60p-NLS-cargo complexes (15). Interestingly, the vertebrate nucleoporin Nup153 also binds importin
α directly at its C terminus (27), where it contains the sequence motif VXRRKXXXXRKR, which resembles the C terminus of Nup1p (Fig. 4A). Thus, vertebrate Nup153 may be functionally analogous to S. cerevisiae Nup1p at their C termini.

Nup2p and Cse1p Couple the Final Step of Kap60p-mediated Nuclear Import with the First Step of Kap60p Export—In summary, we suggest a possible order of events upon entrance of a Kap95p-Kap60p-NLS-cargo complex into the nuclear basket of the yeast NPC. First, the trimeric import complex associates with Kap60p (Fig. 1A, model i) (15). Second, Gsp1p binds to Kap60p in the import complex (7) and accelerates the release of the Kap60p-NLS-cargo complex (15). Third, the Kap60p-NLS-cargo complex binds to the VMXXRKIAXXRKR sequence of Kap60p (Fig. 4B), which in turn accelerates the release of NLS-cargo from Kap60p (Fig. 1A, model i) (15). Fourth, Cse1p in complex with Gsp1p-GTP accelerates the dissociation of Kap60p from Nup2p (Fig. 1A, model ii) (15). Finally, any Kap60p-NLS complexes that escape Nup2p KaRF action would ultimately encounter nucleoplasmic Cse1p, which, together with Gsp1p-GTP, would accelerate release of the NLS while simultaneously capturing Kap60p (Fig. 1A, model iii) (15). In conclusion, Nup2p and Cse1p couple the final step of nuclear import with the first step of Kap60p nuclear export and, together with Gsp1p-GTP, impart directionality to the process.

REFERENCES

1. Mattaj, I. W., and Englmeier, L. (1998) Annu. Rev. Biochem. 67, 265–306
2. Gorlich, D., and Kutay, U. (1999) Annu. Rev. Cell Dev. Biol. 15, 607–660
3. Barry, D. M., and Wente, S. R. (2000) Essays Biochem. 36, 89–103
4. Quimby, B. B., and Corbett, A. H. (2001) Cell Mol. Life Sci. 58, 1766–1773
5. Chook, Y. M., and Blobel, G. (2003) Curr. Opin. Struct. Biol. 11, 715–722
6. Enenkel, C., Blobel, G., and Rexach, M. (1995) J. Biol. Chem. 270, 16499–16505
7. Rexach, M., and Blobel, G. (1995) Cell 83, 683–692
8. Rout, M. P., Aitchison, J. D., Suprapto, A., Hjertaas, K., Zhao, Y., and Chait, B. T. (2000) J. Cell Biol. 148, 635–651
9. Dilworth, D. J., Suprapto, A., Padovan, J. C., Chait, B. T., Wozniak, R. W., Rout, M. P., and Aitchison, J. D. (2001) J. Cell Biol. 153, 1465–1478
10. Solsbacher, J., Maurer, P., Vogel, F., and Schlenstedt, G. (2000) Mol. Cell. Biol. 20, 8468–8479
11. Hood, J. K., Casolari, J. M., and Silver, P. A. (2000) J. Cell Sci. 113, 1471–1480
12. Boger, A. M., Hoffman, J. A., Anbergen, D. C., Fink, G. R., and Davis, L. I. (1994) J. Cell Biol. 127, 319–332
13. Wei, K. (2003) Cell 112, 441–451
14. Dasso, M. (2002) Curr. Biol. 12, 502–508
15. Gilchrist, D., Mykytka, B., and Rexach, M. (2002) J. Biol. Chem. 277, 18161–18172
16. Solsbacher, J., Maurer, P., Bischoff, F. R., and Schlenstedt, G. (1998) Mol. Cell. Biol. 18, 6805–6815
17. Hood, J. K., and Silver, P. A. (1998) J. Biol. Chem. 273, 35142–35146
18. Cate, C., Tew, T., Fontes, M., Jennings, I., Jain, D. A., Howlett, G., Rice, E., and Kore, B. (2001) J. Biol. Chem. 276, 34189–34196
19. Hodel, M. R., Corbett, A. H., and Hodel, A. E. (2001) J. Biol. Chem. 276, 1317–1325
20. Jacobs, L. A., Xiao, C. Y., and Lam, M. H. (2000) BioEssays 22, 532–544
21. Gorlich, D., Pante, N., Kutay, U., Aebi, U., and Bischoff, F. R. (1996) EMBO J. 15, 5584–5594
22. Harreman, M. T., Hodel, M. R., Fanara, P., Hodel, A. E., and Corbett, A. H. (2003) J. Biol. Chem. 278, 5854–5863
23. Harreman, M. T., Cohen, P. E., Hodel, M. R., Truecott, G. J., Corbett, A. H., and Hodel, A. E. (2003) J. Biol. Chem. 278, 21361–21369
24. Ecke, B. (1999) Nat. Struct. Biol. 6, 388–397
25. Allen, N., Huang, L., Burlingame, A., and Rexach, M. (2001) J. Biol. Chem. 276, 29268–29274
26. Denning, D., Mykytka, B., Allen, N., Huang, L., Burlingame, A., and Rexach, M. (2001) J. Cell Biol. 154, 937–950
27. Moretti, J., Blobel, G., and Radu, A. (1997) Proc. Natl. Acad. Sci. U. S. A. 94, 9699–9704
28. Booth, J. W., Belanger, K. D., Sannella, M. I., and Davis, L. I. (1999) J. Biol. Chem. 274, 32360–32367
29. Plumer, M., Blobel, G., and Rexach, M. (1997) J. Biol. Chem. 272, 19538–19546
30. Gruss, O. J., Carazo-Salas, R. E., Schatz, C. A., Guarguaglini, G., Kast, J., Wilm, M., Bot, N., Vernos, I., Karsenti, E., and Mattaj, I. W. (2001) Cell 104, 83–93
31. Lee, J. D., Davis, L. I., and Fink, G. R. (1993) Mol. Biol. Cell 4, 209–222
32. Shulga, N., Roberts, P., Gu, Z., Spitz, L., Tabb, M. M., Nomura, M., and Goldfarb, D. S. (1996) J. Cell Biol. 135, 329–339
33. Schatz, C. A., Santarella, R., Hoeveder, A., Karsenti, E., Mattaj, I. W., Gruss, O. J., and Carazo-Salas, R. E. (2003) EMBO J. 22, 2060–2070
34. Conti, E., Uy, M., Leighton, L., Blobel, G., and Kuriyan, J. (1998) Cell 94, 193–204
35. Kutay, U., Bischoff, F. R., Kostka, S., Kraft, R., and Gorlich, D. (1997) Cell 90, 1061–1071
36. Allen, N. P., Patel, S., Huang, L., Chalkley, R., Burlingame, A., Lutzmann, M., Hurt, E., and Rexach, M. (2002) Mol. Cell Proteomics 1.12, 930–946
37. Lindsay, M. E., Pfaulke, K., Smith, A. E., Clurman, B. E., and Macara, I. G. (2002) Cell 110, 349–369