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Protein Import into Nuclei: Association and Dissociation Reactions Involving Transport Substrate, Transport Factors, and Nucleoporins

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Summary

The molecular dynamics of nuclear protein import were examined in a solution binding assay by testing for interactions between a protein containing a nuclear localization signal (NLS), the transport factors karyopherin α, karyopherin β, and Ran, and FXFG or GLFG repeat regions of nucleoporins. We found that karyopherins α and β cooperate to bind FXFG but not GLFG repeat regions. Binding of the NLS protein to karyopherin α was enhanced by karyopherin β. Two novel reactions were discovered. First, incubation of a karyopherin heterodimer-NLS protein complex with an FXFG repeat region stimulated the dissociation of the NLS protein from the karyopherin heterodimer. Second, incubation of the karyopherin heterodimer with RanGTP (or with a Ran mutant that cannot hydrolyze GTP) led to the dissociation of karyopherin α from β and to an association of Ran with karyopherin β; RanGDP had no effect. We propose that movement of NLS proteins across the nuclear pore complex is a stochastic process that operates via repeated association-dissociation reactions.

Introduction

Import of proteins that contain a nuclear localization signal (NLS proteins) across the nuclear pore complex (NPC) has been proposed to proceed by guided diffusion involving multiple docking sites in nucleoporins (a collective term for NPC proteins) that constitute a stationary phase, as well as soluble transport factors that function as a mobile phase (Radu et al., 1995a). This hypothesis is rooted in two observations. First, gold-labeled NLS proteins injected into the cytoplasm of amphibian oocytes are seen at multiple sites along the cytoplasmic fibers, the central transporter, and nucleoplasmic baskets of the NPC before they accumulate in the nucleoplasm (Feldherr et al., 1984; Richardson et al., 1988). Second, several nucleoporins that contain regions with repetitive peptide motifs were identified as potential docking sites using overlay blot assays (Radu et al., 1995a, 1995b). As members of this family of nucleoporins reside throughout the cytoplasmic fibrils, the central transporter, and the nucleoplasmic baskets of the NPC (reviewed by Rout and Wente, 1994), it was proposed that nucleoporin repeats are the biochemical correlates of the multiple docking sites observed by electron microscopy and that these multiple docking sites guide the saltatory movement of karyopherin–NLS protein complexes from the cytoplasmic to the nucleoplasmic side of the NPC by a series of docking and undocking reactions (Radu et al., 1995a).

Docking of NLS proteins to the NPC, as well as their subsequent movement across the NPC, requires transport factors. Using an in vitro assay that reconstitutes import of a transport substrate into nuclei of digitonin-permeabilized cells (Adam et al., 1990), four such factors have been purified from cytosol. These are the GTPase Ran (Moore and Blobel, 1993; Melchior et al., 1993), the Ran-interacting protein p10 (Moore and Blobel, 1994; Paschal and Gerace, 1995), and a heterodimeric complex termed karyopherin (Radu et al., 1995b; Moroianu et al., 1995a; Enenkel et al., 1995), or NLS receptor (Adam and Gerace, 1991; Weis et al., 1995) and p97 (Adam and Adam, 1994; Chi et al., 1995), or importin 60 (Görlich et al., 1994) and importin 90 (Görlich et al., 1995a), or nuclear pore–targeting complex (Imamoto et al., 1995a, 1995b). Karyopherin α recognizes the NLS of the transport substrate (Adam and Gerace, 1991; Moroianu et al., 1995a, 1995b; Weis et al., 1995), whereas karyopherin β mediates docking of karyopherin α–NLS protein complexes to nucleoporins that contain peptide repeat regions (Radu et al., 1995a, 1995b; Moroianu et al., 1995b). Ran and p10 are required for the movement of the docked NLS protein into the nucleoplasm (Moore and Blobel, 1993, 1994; Radu et al., 1995b). The cytosol requirement in reconstituted nuclear import reactions is replaced using recombinant human karyopherins, Ran, and p10 (Moroianu et al., 1995b), but the molecular dynamics of substrate movement across the NPC and the functional relationship between karyopherin heterodimers, Ran, p10, and nucleoporins remain to be elucidated.

The mechanism of protein import is comparable in yeast, as its NPC is similar in structure and composition to that of vertebrates (Rout and Blobel, 1993) and as homologs of karyopherins α and β, Ran, and p10 have been identified. The yeast homolog of karyopherin α is essential for cell growth and was originally named Srp1 (for supressor of a mutation in a subunit of RNA polymerase I; Yano et al., 1992), but was renamed Kap60 for karyopherin of 60 kDa (Enenkel et al., 1995) to avoid confusion with the previously issued acronym SRP (signal recognition particle; Walter and Blobel, 1982). Kap60 exists in yeast cytosol in a complex with Kap95, which is an essential homolog of vertebrate karyopherin β (Enenkel et al., 1995). Recombinant Kap60 and Kap95 assemble into a heterodimer that functions to dock NLS proteins to nuclear envelopes in digitonin-permeabilized mammalian cells (Enenkel et al., 1995). Yeast has an essential homolog of Ran named Gsp1 (Belhumeur et al., 1993; Kadowaki et al., 1994), as well as a homolog of p10 that was recently identified in the yeast genome sequencing project, but further characterization has not yet been reported.

To understand better the dynamics of interaction between components of the stationary phase (nucleoporins) and the mobile phase of transport (transport factors and substrate), we turned to solution binding assays using recombinant yeast karyopherin α (Kap60), karyopherin β

(Kap95), and Ran (Gsp1), recombinant FXFG repeat regions of yeast nucleoporins Nup1 and Nup2, recombinant GLFG repeat regions of yeast nucleoporins Nup145 and Nup57, and a recombinant NLS protein (glutathione S-transferase [GST] with a C-terminal NLS, termed GST-NLS). As expected, the NLS protein bound to karyopherin α. Karyopherin β bound to α and increased its affinity for the NLS protein. Surprisingly, addition of a soluble FXFG repeat region to the assembled karyopherin heterodimer–NLS protein complex stimulated the dissociation of the NLS protein from the karyopherin heterodimer, accompanied by binding of the karyopherin heterodimer to the FXFG repeat region. Most strikingly, RanGTP, RanGMPPcP, or mutant Ran that cannot hydrolyze GTP promoted the disruption of the karyopherin heterodimer by binding directly to karyopherin β and caused the release of both karyopherin subunits from the docking site.

Results

We used a solution binding assay to examine the interactions between Kap60 (yeast karyopherin α), Kap95 (yeast karyopherin β), Gsp1 (yeast Ran), a recombinant NLS protein (GST-NLS), and recombinant repeat regions of Nup1, Nup2, Nup57, and Nup145 (yeast nucleoporins). These proteins (except for Gsp1) were expressed as GST fusions that bind to glutathione–agarose beads in solution. As all GST fusions contained a thrombin cleavage site at the chimeric junction, the GST portions were removable by proteolysis. Only one GST chimera was retained and immobilized in each binding assay. The bound and unbound proteins were visualized by SDS–polyacrylamide gel electrophoresis (SDS–PAGE) and Comassie blue staining; hence, the sensitivity of the binding assays allowed detection of those interactions that were stable and of high affinity but not of those that were unstable and of low affinity.

Karyopherin Heterodimers Bind to Nucleoporin FXFG but Not GLFG Repeat Regions

Peptide repeat regions in nucleoporins contain multiple copies of one or more types of tetrapeptide motifs. Most noticeable are the FXFG and the GLFG types (reviewed by Rout and Wente, 1994). FXFG repeat regions contain numerous clusters of acidic and basic amino acids flanking the FXFG repeats, whereas GLFG repeat regions lack acidic amino acids but contain numerous clusters of asparagine and glutamine residues flanking the GLFG repeats. To determine whether karyopherins bind equally well to the distinct peptide repeat regions, we expressed portions of yeast nucleoporins as GST chimeras that contain only one type of repeat region. We then tested the ability of yeast karyopherin subunits Kap60 and Kap95 to bind these chimeras immobilized on glutathione–agarose beads. We found that karyopherin heterodimers bind to the FXFG repeat region of Nup1 and Nup2, but not to the GLFG repeat region of Nup145 or Nup57 (Figure 1). Tight binding of Kap60 and Kap95 to the FXFG repeat region of Nup1 (Figure 1A, lane 3) and Nup2 (data not shown) was detected only when the Kap proteins were combined (Figures 1A and 1B, lanes 3) under conditions that allow

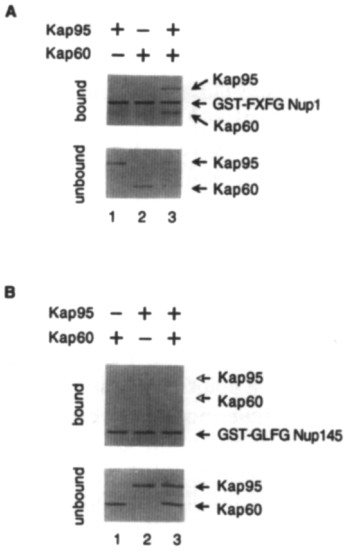

Figure 1. The Karyopherin Heterodimer Binds to the FXFG Repeat Region of Nucleoporin Nup1 but Not to the GLFG Repeat Region of Nucleoporin Nup145

Immobilized GST-FXFG Nup1 (A) or GST-GLFG Nup145 (B) (0.6 μg of each per 10 μl of packed beads) was incubated for 45 min at 20°C with Kap60 (0.6 μg), or Kap95 (0.6 μg), or both. Bound and unbound fractions were analyzed by SDS–PAGE and Coomassie blue staining.

their spontaneous assembly into a heterodimer (Enenkel et al., 1995). Karyopherin heterodimers bound to the FXFG region of Nup1 with higher affinity than to the FXFG region of Nup2 (data not shown). Karyopherin heterodimers did not bind to the GLFG repeat region of Nup145 (Figure 1B, lane 3) or Nup57 (data not shown). Neither Kap95 nor Kap60 monomers bound tightly to any of the repeat regions (Figures 1A and 1B, top panels, lanes 1 and 2; data not shown). Weak binding of Kap95 but not Kap60 monomers to FXFG repeat regions of Nup1 and Nup2 was detected using silver staining, a more sensitive method of detection (data not shown). These data suggest that karyopherin subunits cooperate to bind nucleoporin FXFG repeat regions.

Assembly and Disassembly of a Karyopherin Heterodimer-NLS Protein Complex

To determine whether Kap60, like vertebrate karyopherin α, binds to an NLS protein, we expressed the NLS of the large T antigen linked in a C-terminal fusion to GST to generate GST-NLS. We then tested the ability of Kap60 and Kap95 to bind GST-NLS immobilized on glutathione–agarose beads (Figure 2A). We found that Kap60 monomers bound the NLS protein (Figure 2A, lane 2) and that Kap95 monomers did not (lane 1, compare bound versus unbound). However, addition of increasing amounts of Kap95 resulted in a corresponding increase in the binding of Kap60 to the NLS protein (Figure 2A, lanes 3–5). The binding of Kap60 to the NLS protein was enhanced when Kap95 formed a complex with Kap60 (Figure 2A, compare lanes 2 and 5). Once again, the karyopherin subunits cooperated to bind a ligand, in this case the NLS. Binding was specific as karyopherin heterodimers did not bind to GST-

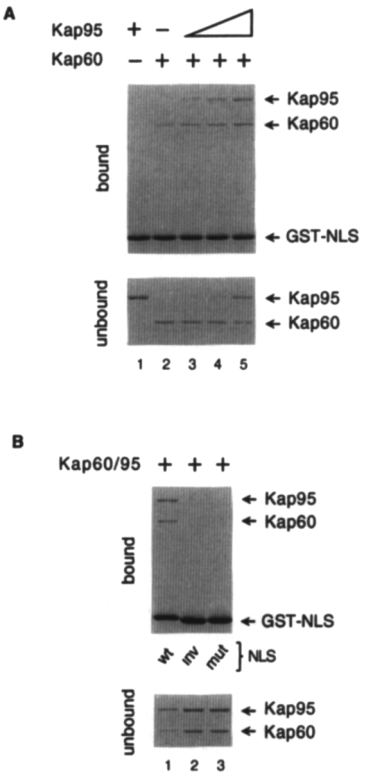

Figure 2. Karyopherin α (Kap60) Binds to a Recombinant Protein That Contains a Functional NLS (GST-NLS), and Binding Is Enhanced by Recruitment of Karyopherin β (Kap95)

(A) Immobilized GST-NLS (1 μg per 10 μl of packed beads) was incubated for 45 min at 20°C with Kap95 (0.6 μg) (lane 1) or Kap60 (0.6 μg) (lane 2), or Kap60 (0.6 μg) and increasing amounts of Kap95 (0.15, 0.3, and 0.6 μg) (lanes 3, 4, and 5).

(B) Immobilized GST-NLS (wt) (lane 1), GST-NLS inverse (inv) (lane 2), or GST-NLS mutant (mut) (lane 3) (1 μg per 10 μl of packed beads) was incubated for 45 min at 20°C with Kap95 and Kap60 (0.6 μg of each). Bound and unbound fractions were analyzed by SDS–PAGE and Coomassie blue staining.

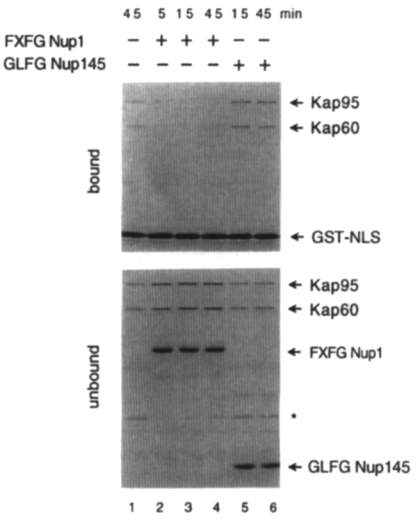

Figure 3. The FXFG Repeat Region of Nup1 Stimulates the Release of an NLS Protein from the Karyopherin Heterodimer

Immobilized GST-NLS (1 μg per 10 μl of packed beads) was preincubated for 45 min at 4°C with Kap60 (0.6 μg) and Kap95 (0.6 μg). After washing, the beads were incubated for 45 min at 20°C with no addition (lane 1), or were incubated for various times (5 min, 15 min, 45 min) with the FXFG repeat region of Nup1 (0.6 μg) (lanes 2–4) or with the GLFG repeat region of Nup145 (0.6 μg) (lanes 5 and 6). Bound and unbound fractions were analyzed by SDS–PAGE and Coomassie blue staining. The star marks GST-NLS that detached from beads during incubation.

NLS chimeras that contained the inverse version of the large T antigen NLS (NLS-inv) (Figure 2B, lane 2) or the mutant version (NLS-mut) (Figure 2B, lane 3), which is not functional for import in vivo or in vitro (Kalderon et al., 1984; Adam et al., 1990). The observed cooperation between Kap60 and Kap95 in binding NLS proteins and nucleoporin FXFG repeat regions is consistent with their functional synergism in targeting an import substrate to the nuclear envelope of digitonin-permeabilized mammalian cells (Enenkel et al., 1995).

In an attempt to reconstitute a stable docking complex composed of the NLS protein, the karyopherin heterodimer, and a nucleoporin FXFG repeat region, we allowed binding of karyopherin heterodimers to GST-NLS chimeras as in Figure 2 and subsequently added a soluble Nup1 FXFG repeat region (the same one used in Figure 1A but without the GST portion). Surprisingly, addition of the FXFG repeat region stimulated the release of karyopherin heterodimers from the GST-NLS chimera (Figure 3, lanes 2–4, compare with lane 1). Addition of a soluble Nup145 GLFG repeat region (the same one used in Figure 1B but without the GST portion) had no effect (Figure 3, lanes 5 and 6). These results combined with the results in Figure 1 suggest that the karyopherin heterodimer–GST-NLS complex binds to the FXFG repeat region in a configuration that stimulates the release of the NLS protein from Kap60. Indeed, we could not detect binding of a different NLS protein to karyopherin heterodimer–GST-FXFG repeat region complexes immobilized on glutathione–agarose beads even though this NLS protein would bind efficiently to an immobilized Kap60–GST-Kap95 complex (data not shown). Addition of a soluble NLS peptide (CYTPPKKKRKV) prevented binding of karyopherin heterodimers to the GST-NLS chimera but not to the FXFG repeat region of Nup1 (data not shown).

RanGTP Dissociates Karyopherin Subunits from Docking Sites

When recombinant yeast Ran was added to karyopherin heterodimer–FXFG repeat region complexes assembled as described in Figure 1A, there was release of both karyopherin subunits from the repeat region (Figure 4A, lanes 3–5, compare with lane 1). In a similar experiment, coincubation of karyopherin heterodimers with Ran prevented the binding of karyopherins to immobilized FXFG repeat regions (data not shown). As the added Ran consisted of a mixture of the GTP-bound, the GDP-bound, and the unbound forms (M. Floer and G. B., unpublished data), we sought to determine whether these forms of Ran have distinct effects in the dissociation of karyopherins from the FXFG repeat region. Ran was first preincubated with

EDTA and either GTP, GDP, GMPPcP, or no nucleotide, and then with $Mg(OAc)_2$ to promote binding of the added nucleotide to Ran. The GTP-preincubated Ran was functional in causing the dissociation of both karyopherin subunits from the repeat region (Figure 4B, lane 3, compare with lane 1), whereas the GDP-preincubated Ran was not (lane 4). Interestingly, the GMPPcP-preincubated Ran was effective in dissociating the karyopherin subunits from the FXFG repeat region (Figure 4B, lane 5), indicating that GTP hydrolysis is not required for this reaction. The absence of added nucleotide during the preincubation reaction inactivated Ran (Figure 4B, lane 6, compare with lane 3). Ran did not bind directly to the FXFG repeat region (data not shown).

RanGTP Disrupts the Karyopherin Heterodimer via Binding to Karyopherin β

Addition of Ran to Kap60–GST-Kap95 complexes that were immobilized on glutathione–agarose beads caused the dissociation of Kap60 from GST-Kap95 (Figure 5A, lane 2, compare with lane 1). A fraction of the added Ran remained bound to GST-Kap95 (Figure 5A, lane 2). To determine the nucleotide requirement of Ran in this association–dissociation reaction, Ran was preincubated with different guanine nucleotides as before. RanGTP (Figure 5A, lane 3) and RanGMPPcP (lane 5) bound tightly to GST-Kap95 and caused the dissociation of Kap60; RanGDP did not (lane 4). RanGTP bound directly to immobilized GST-Kap95, even in the absence of Kap60 (data not shown); Ran did not bind to immobilized GST-Kap60 (data not shown). Experiments with a mutant isoform of human Ran that can bind but not hydrolyze GTP (mutant Ran) were done to further support our conclusion that GTP hydrolysis is not required for the Ran-dependent disruption of the karyopherin heterodimer. Addition of the wild-type version of human Ran (wild-type Ran) to Kap60–GST-Kap95 complexes immobilized on glutathione–agarose beads caused the dissociation of Kap60 from GST-Kap95 (Figure 5B, lane 2, compare with lane 1); a fraction of the added wild-type RanGTP remained bound to GST-Kap95 (lane 2). In contrast, wild-type RanGDP had no effect (lane 3). Addition of GTP or GDP to Kap60–GST-Kap95 complexes had no effect (data not shown). Mutant RanGTP was also functional in causing the dissociation of Kap60 from GST-Kap95 (Figure 5B, lane 4, compare with lane 2), and a fraction of mutant RanGTP also remained bound to GST-Kap95 (lane 4). Mutant RanGDP was not functional in dissociating the karyopherin subunits, yet a small fraction bound to GST-Kap95 (Figure 5B, compare lane 5 with lane 1), implying that Kap60 and Ran do not compete for the same binding site in Kap95. The faint protein band in lane 1 (marked by the star) that comigrated with human Ran is GST and was a contaminant present in equal amounts in the lanes (see also Figure 5A). Based on these results, we concluded that RanGTP disrupts the karyopherin heterodimer by binding to karyopherin β and that Ran does not hydrolyze GTP to perform this function. The RanGTP-dependent disruption of the karyopherin heterodimer causes the dissociation of both karyopherin subunits from the FXFG repeat region (Figure 4) because neither subunit alone can bind this region with high affinity (see Figure 1).

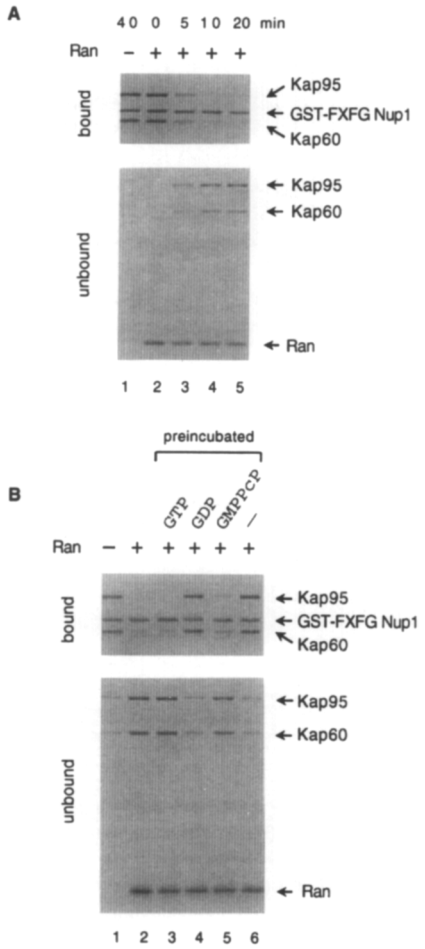

Figure 4. The GTP-Bound Form of Ran Stimulates the Release of Karyopherin Subunits from the FXFG Repeat Region of Nup1

Immobilized GST-FXFG Nup1 (0.6 μg per 10 μl of packed beads) was preincubated for 30 min at 20°C with Kap60 and Kap95 (0.6 μg each). After washing, the beads were incubated at 20°C for 40 min with no addition (lane 1), or for 0, 5, 10, and 20 min with Ran (0.6 μg) (lanes 2–5) (A); the beads were incubated at 20°C for 30 min with no addition (lane 1), or with Ran (0.6 μg) (lane 2), or with Ran (0.6 μg) that had been preincubated with GTP (lane 3), GDP (lane 4), GMPPcP (lane 5), or no nucleotide (lane 6) (B). Bound and unbound fractions were analyzed by SDS–PAGE and Coomassie blue staining.

The finding that RanGTP causes the disruption of the karyopherin heterodimer predicted that addition of RanGTP to a karyopherin heterodimer–NLS protein complex would lead to the dissociation of karyopherin β from α and to a concomitant loss of affinity of karyopherin α for the NLS protein, as this affinity is weaker in the absence of bound karyopherin β (see Figure 2). Indeed, addition of RanGTP to a karyopherin heterodimer–GST-NLS complex stimulated the dissociation of karyopherins β and α from the NLS protein (Figure 6, lane 2, compare with lane 1). Addition of RanGDP had no effect (Figure 6, lane 3).

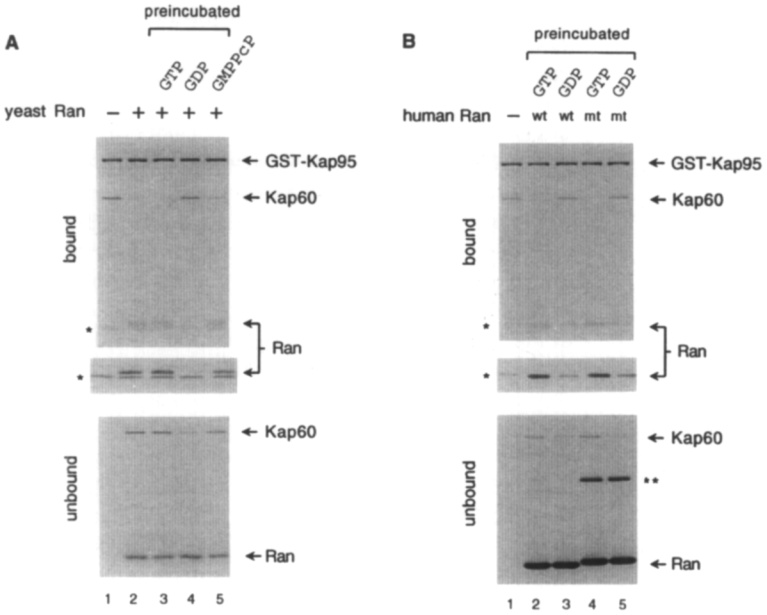

Figure 5. The GTP-Bound Form of Ran Disrupts the Karyopherin Heterodimer via Binding to Karyopherin β (Kap95)

Immobilized GST-Kap95 (0.6 μg per 10 μl of packed beads) was preincubated for 20 min at 20°C with Kap60 (0.6 μg). After washing, the beads were incubated for 30 min at 20°C with no addition (lane 1), or with yeast Ran (0.6 μg) (lane 2), or with yeast Ran (0.6 μg) that had been preincubated with GTP (lane 3), GDP (lane 4), or GMPPcP (lane 5) (A); the beads were incubated for 30 min at 20°C with no addition (lane 1), or with human Ran (2 μg) that had been preincubated with GTP (lane 2) or GDP (lane 3), or with mutant human Ran that had been preincubated with GTP (lane 4) or GDP (lane 5) (B). Bound and unbound fractions were analyzed by SDS–PAGE and Coomassie blue staining (top and bottom panels) and silver staining (middle panel). The star marks GST (a contaminant in this case). The double star marks mutant Ran that dimerizes during preparation for SDS–PAGE.

Discussion

Our solution binding assay revealed several association–dissociation reactions between components of the stationary phase (nucleoporin FXFG repeat regions) and the mobile phase (transport factors and NLS proteins) of transport across the NPC. The interactions between these proteins are illustrated in Figure 7 and are summarized as follows.

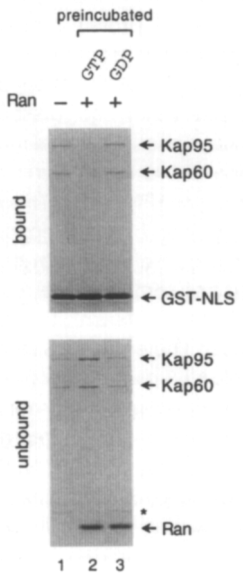

Figure 6. The GTP-Bound Form of Ran Stimulates the Dissociation of an NLS Protein from the Karyopherin Heterodimer

Immobilized GST-NLS (1 μg per 10 μl of packed beads) was preincubated for 45 min at 4°C with Kap60 and Kap95 (0.6 μg of each). After washing, the beads were incubated for 45 min at 20°C with no addition (lane 1) or with Ran (0.6 μg) that had been preincubated with GTP (lane 2) or GDP (lane 3). Bound and unbound fractions were analyzed by SDS–PAGE and Coomassie blue staining. The star marks GST-NLS that detached from beads during incubation.

Karyopherin α (Kap60) binds an NLS protein (Figure 2A). Karyopherin β (Kap95) binds to karyopherin α and enhances its affinity for the NLS protein (Figure 2A). Karyopherins α and β do not require an NLS protein to assemble spontaneously into a heterodimer (Figure 5; Enenkel et al., 1995). When a soluble karyopherin heterodimer–NLS protein complex encounters a nucleoporin that contains an FXFG repeat region, the karyopherin heterodimer binds to this region (Figure 1) in a reaction that lowers the affinity of the NLS protein for karyopherin (Figure 3). The karyopherin heterodimer–FXFG repeat region complex is disrupted by Ran (Figure 4). RanGTP but not RanGDP functions to break apart the karyopherin heterodimer by forming a complex with karyopherin β (Figure 5) and does not utilize GTP hydrolysis to perform this function (Figure 5). RanGTP and karyopherin β form a heterodimer (M. Floer and G. B., unpublished data). The Ran-dependent disruption of the karyopherin heterodimer stimulates the release of both karyopherin subunits from the FXFG repeat region (Figure 4) because neither subunit alone has a high affinity to this region (Figure 1A). It is likely that additional factors that were not tested in the binding assay (i.e., p10, the Ran-binding protein RanBP1, and Ran-specific GAPs and GEFs) modify these association–dissociation reactions. We propose that nuclear protein import occurs by repeated cycles of the observed association and dissociation reactions. These reactions may occur at every location of the NPC (cytoplasmic, central, and nucleoplasmic) in which there is an exposed nucleoporin FXFG repeat region. The repeated assembly and disassembly of karyopherin-mediated docking complexes throughout the NPC may function to generate a high local concentration of NLS proteins that facilitate the "guided diffusion" of NLS proteins across a central channel in a stochastic process that entails the saltatory movement of NLS proteins and karyopherin from one docking site to another. We speculate that the directionality of nuclear protein import is ulti-

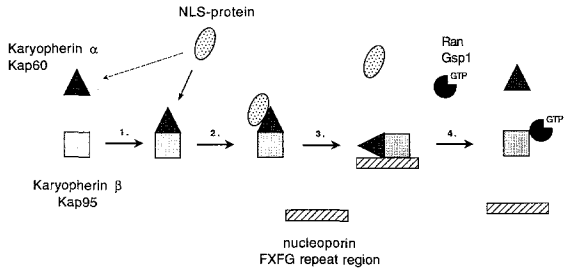

Figure 7. Partial Reactions in a Cycle of Karyopherin-Mediated Docking of NLS Protein to the FXFG Repeat Region of a Nucleoporin

Karyopherin subunits assemble spontaneously into a heterodimer (step 1). A NLS protein binds with low affinity (indicated by the dashed arrow) to karyopherin α monomers (Kap60), or with high affinity (indicated by the solid arrow) to karyopherin α in a karyopherin heterodimer (step 2). When the karyopherin heterodimer–NLS protein complex encounters a nucleoporin FXFG repeat region, the karyopherin heterodimer binds to this region and releases the NLS protein (step 3). The GTP bound form of Ran (Gsp1) causes the release of karyopherin subunits from the FXFG repeat region by forming a complex with karyopherin β (Kap95) (step 4).

mately achieved not by an intrinsic mechanism for directional movement of proteins in the NPC, but by the presence of anchoring sites in the nucleoplasm that capture and retain NLS proteins that traverse the NPC.

The binding of yeast karyopherin α/β complexes to the NLS protein via the α subunit (Figure 2) is consistent with previously reported data as follows: karyopherin α/β complexes can be isolated from cytosol (Radu et al., 1995b; Görlich et al., 1995a; Imamoto et al., 1995a; Enenkel et al., 1995); NLS proteins bind directly to karyopherin α (Adam and Gerace, 1991; Moroianu et al., 1995a, 1995b; Weis et al., 1995) and not to β (Moroianu et al., 1995a); karyopherin α and β subunits are required in combination for docking of NLS proteins at the nuclear rim of digitonin-permeabilized cells (Adam and Adam, 1994; Görlich et al., 1995a; Moroianu et al., 1995a, 1995b; Enenkel et al., 1995). The novel aspect of our results is that the karyopherin α/β heterodimer has a higher affinity for the NLS protein than karyopherin α alone (Figure 2A); this suggests that karyopherin β increases the affinity of karyopherin α for the NLS protein. Alternatively, karyopherin β may trigger the exposure of additional NLS binding sites in karyopherin α, or karyopherin α may trigger the exposure of an NLS-binding site(s) in karyopherin β. The observed binding of the karyopherin α/β heterodimer to the NLS protein was specific as karyopherin heterodimers did not bind the NLS protein when its NLS was modified by reversal of the amino acid sequence that encodes it, or by substitution of one charged amino acid (Figure 2B) that renders the NLS not functional for import in vivo and in vitro (Kalderon et al., 1984; Adam and Gerace, 1991).

Karyopherin heterodimers bound to the FXFG repeat region of Nup1 (Figure 1A) and Nup2 (data not shown), but not to the GLFG repeat region of Nup145 (Figure 1B) or Nup57 (data not shown). These findings suggest that karyopherin heterodimers bind to nucleoporin FXFG repeat regions but not to GLFG repeat regions. Data from blot overlay assays support this observation, in that those nucleoporins that have been shown to bind karyopherin contain an FXFG repeat region (Radu et al., 1995b; Kraemer et al., 1995). The apparent preference of karyopherin heterodimers for FXFG repeat regions suggests an alternate role for GLFG repeat regions. Both vertebrates and yeast have at least five nucleoporins that contain an FXFG repeat region (reviewed by Rout and Wente, 1994). It is commonly assumed that each nucleoporin exists in multiple copies per NPC (an average of 8–16 copies); hence, there could be more than 40–80 potential docking sites distributed throughout the cytoplasmic fibers, the central transporter, and nucleoplasmic baskets of the NPC. How does the karyopherin heterodimer contact the FXFG repeat region? Although we observed in the solution binding assay that neither karyopherin α nor β monomers bound tightly to the FXFG repeat regions, weak binding of karyopherin β but not α was detected using silver staining, a more sensitive method of detection (data not shown). Thus, the model shown in Figure 7 indicates that karyopherin α/β binds to the FXFG repeat region via karyopherin β. This assignment is confirmed by previously reported data that karyopherin β but not α monomers bound directly to the nuclear rim of digitonin-permeabilized cells (Moroianu et al., 1995b; Görlich et al, 1995b) and that radiolabeled karyopherin β bound directly to nucleoporins that contain FXFG repeat regions in a blot overlay assay (Moroianu et al., 1995b). We do not exclude the possibility that karyopherin α contacts the FXFG repeat region when bound to karyopherin β. It remains to be determined whether the karyopherin heterodimer binds directly to the FXFG peptide motif, or to the surrounding charged region, or to both.

Addition of a nucleoporin FXFG repeat region to a karyopherin heterodimer–NLS protein complex stimulated the dissociation of NLS protein from the karyopherin heterodimer (Figure 3). A coupled association–dissociation reaction explains this observation best: dissociation of the NLS protein from the karyopherin heterodimer (Figure 3) coupled to association of the karyopherin heterodimer with the FXFG repeat region (Figure 1). This coupled reaction was specific, as addition of a nucleoporin GLFG repeat region or ovalbumin, both of which do not bind karyopherin heterodimers, did not stimulate the release of the NLS protein (Figure 3; data not shown). The karyopherin heterodimer did not bind to a potential NLS sequence in Nup1 as addition of a soluble NLS peptide that prevented binding of karyopherin to the NLS protein did not affect binding to the FXFG repeat region of Nup1 (data not shown). Karyopherin heterodimers may release NLS proteins at the NPC simply by docking to nucleoporins that contain FXFG repeat domains. Lowering the affinity of the karyopherin heterodimer for the NLS protein is indeed an efficient method of returning the NLS protein to the mobile phase of transport after docking to any of multiple FXFG repeat regions stationed along the NPC. This coupled association–dissociation reaction was surprising, as it appears to contradict the observed karyopherin-mediated docking of a fluorescent NLS protein to the nuclear rim of permeabilized cells (Adam and Adam, 1994; Radu et al., 1995b; Görlich et al., 1995a; Moroianu et al., 1995a, 1995b; Imamoto et al., 1995a; Enenkel et al., 1995). There are at

least two possible explanations. First, our solution binding assay scores as dissociation/release a decrease in binding affinity to levels that are not detected in the assay; these low affinity interactions may be sufficient to detect binding by fluorescence microscopy in the digitonin-permeabilized cell assay. Second, additional components of the stationary or mobile phase of transport that were not included in the solution binding assay may function to stabilize the interaction between the NLS protein and karyopherin heterodimer bound to the FXFG repeat region.

The most important coupled association–dissociation reaction detected in the solution binding assay is the RanGTP-induced dissociation of karyopherin α from karyopherin β and the concomitant association of RanGTP with karyopherin β (Figure 5). This coupled reaction is specific, as RanGDP, which does not bind tightly to karyopherin β, does not cause the dissociation of karyopherin subunits. The RanGTP-dependent dissociation of karyopherin heterodimers appears to be an obligatory step for transport of NLS proteins beyond docking sites in the NPC, as karyopherin α and the NLS protein accumulate in the nucleus during import reactions, whereas karyopherin β does not (Moroianu et al., 1995b; Görlich et al., 1995b). We observed that the Ran-dependent disruption of the karyopherin heterodimer weakens the interaction between the karyopherin heterodimer and the NLS protein (Figure 6) and stimulates the release of both karyopherin subunits from the docking site(s) in the FXFG repeat region (Figure 4) so as to regenerate the site for another round of karyopherin-mediated binding and release of an NLS protein (Figure 7). In essence, RanGTP imparts fluidity to the mobile phase of transport, as disruption of the karyopherin heterodimer serves to relegate the NLS protein, karyopherin α, and karyopherin β back to the mobile phase to initiate another cycle of NLS protein binding, docking, and release. However, karyopherin β could repeat another cycle of docking only after its bound RanGTP is released; this could be accomplished by GTP hydrolysis or by exchange of GTP for GDP as RanGDP does not bind tightly to karyopherin β (Figure 5). We would expect that the levels of RanGTP in the cytosol are normally kept very low, because formation of RanGTP–karyopherin β complexes in the cytosol would be deleterious to the cell, as these complexes would sequester karyopherin β into a form that cannot form heterodimers and hence could not function in docking NLS proteins to nucleoporin FXFG repeat regions (Figures 5 and 6). Cells contain a Ran-specific GAP in the cytoplasm that functions alone and in synergism with another cytosolic protein (RanBP1) to convert cytosolic RanGTP to RanGDP (Bischoff et al., 1995). These observations suggest that RanGTP is generated only locally at the NPC where its function is beneficial. Indeed, RanGTP is generated from RanGDP in a reaction that requires p10 and karyopherin heterodimers bound to a nucleoporin FXFG repeat region (U. Nehrbass and G. B., unpublished data).

Direct evidence that GTP hydrolysis by Ran is necessary to sustain the continuous traffic of proteins across the NPC comes from experiments in yeast in which a mutant form of Ran that binds but does not hydrolyze GTP blocks nuclear protein import and mRNA export in vivo (Schlenstedt et al., 1995). In addition, nonhydrolyzable analogs of GTP can inhibit the movement of NLS proteins into the nucleoplasm of permeabilized cells (Moore and Blobel, 1993; Melchior et al., 1993). To determine whether Ran hydrolyzes GTP to function in the association–dissociation reactions identified here, we preincubated Ran with two different nonhydrolyzable analogs of GTP. Yeast Ran was functional in disrupting karyopherin heterodimers when preincubated with GMPPcP (Figure 5) but not with GMPPnP (data not shown). This apparent discrepancy may have resulted from an inability of yeast Ran to bind GMPPnP during the preincubation reaction, as the absence of added nucleotide during preincubation inactivates yeast Ran (Figure 5, lane 6). Alternatively, the structure of yeast Ran when bound by GMPPnP may not be compatible for binding karyopherin β; this peculiarity may only be true for yeast Ran.

To confirm that GTP hydrolysis is not required for the Ran-dependent disruption of the karyopherin heterodimer, we used a mutant form of human Ran that can bind but not hydrolyze GTP. The mutant and wild-type forms of human RanGTP were functional in causing the dissociation of karyopherin α from β (Figure 5B) and associated with karyopherin β. The reaction was specific as the mutant and wild-type forms of Ran did not cause the dissociation of the karyopherin subunits when preincubated with GDP instead of GTP. Based on these results and those obtained with GMPPcP, we concluded that Ran does not hydrolyze its bound GTP to function in the disruption of the karyopherin heterodimer. GTP hydrolysis by Ran may instead be required for its dissociation from karyopherin β, which would be essential to recycle β for another cycle of docking and undocking. If repeated cycles of docking and undocking are required to move NLS proteins from sites on the cytoplasmic side of the NPC to sites on the nucleoplasmic side, then incubation of Ran and a nonhydrolyzable analog of GTP with nuclei that contain NLS proteins docked at the cytoplasmic side of the NPC would ultimately result in the release of the NLS protein from NPCs. Indeed, import of NLS proteins that had been docked to the nuclear rim of digitonin-permeabilized cells did not proceed in a subsequent incubation with Xenopus Ran, p10, and GMPPnP; instead the docked transport substrate was found to be released from the nuclear rim (Moore and Blobel, 1994).

In conclusion, we discovered two novel dissociation–association reactions that are likely to be key events in protein import into nuclei. One is the nucleoporin-induced dissociation of transport substrate from the karyopherin heterodimer; the other is the RanGTP-dependent disruption of the karyopherin heterodimer into monomers via binding to karyopherin β.

Experimental Procedures

Solution Binding Assay

All assays were performed using recombinant proteins (see below) in binding buffer (20 mM HEPES [pH 6.8], 150 mM KOAc, 2 mM $Mg(OAc)_2$, 2 mM DTT, 0.1% Tween 20, and 0.1% casaminoacids). Tween (Sigma) and casaminoacids (Difco Laboratories) were used as blocking agents to prevent nonspecific aggregations. For each experi-

ment, the GST chimera was incubated in batch with glutathione–agarose beads (0.6–1.0 μg of GST fusion per 10 μl of packed beads) (Sigma) in 0.5 ml of binding buffer for 45 min at 4°C or 20 min at room temperature. The beads were collected by centrifugation at 2000 × g for 30 s and were washed three to six times by resuspension in 0.5 ml of binding buffer and sedimentation as before. Washed collected beads were resuspended in a 50% slurry by adding 1 vol of binding buffer.

One-Step Assay

The bead slurry was aliquoted in 20 μl portions into siliconized 0.5 ml microtubes (Sigma) that contained protein additions, for a total volume of 40 μl, and were then tumbled end over end for 45 min at room temperature.

Two-Step Assay

The bead slurry was incubated in batch with Kap60 and/or Kap95 (0.6 μg of each for every 10 μl of packed beads) for 30 min at room temperature or 45 min at 4°C in a volume of 40 μl for every 10 μl of packed beads. After washing two to three times each with 0.5 ml of binding buffer as before, the beads were resuspended in a 50% slurry and incubated for different times with protein additions, as in the one-step assay. At the end of incubations, beads were collected by centrifugation at 2000 × g for 30 s, and unbound proteins in the supernatant were collected by removing 28 μl from the meniscus: this constitutes the unbound fraction. Beads were washed twice by mixing with 0.5 ml of binding buffer at room temperature followed by sedimentation as before and were resuspended with 20 μl of buffer. All samples were finally processed by adding 10 μl of 6× sample buffer with β-mercaptoethanol and heating at 95°C for 10 min. Proteins in one half of each sample were resolved by SDS–PAGE and stained with Coomassie brilliant blue (Bio-Rad) or silver.

Preparation of Recombinant Karyopherin Subunits

Yeast karyopherins α and β were expressed separately as GST fusion proteins (Pharmacia) in the protease-deficient Escherichia coli strain BLR (Novagen) and were purified from E. coli lysates on glutathione–agarose beads as described previously (Enenkel et al., 1995), or as described for the recombinant nucleoporins (see below). The purified proteins were dialyzed extensively against binding buffer (without blocking agents) to remove the reduced glutathione used for elution from the affinity beads. Portions of each purified chimera were aliquoted, frozen in liquid nitrogen, and stored at −70°C. As the fusion proteins contained a thrombin site at the chimeric junction, the GST portion of the chimera was cleaved by a 10–30 min incubation at room temperature with 1.5 NIH units of thrombin (Sigma) per 100 μg of chimera. GST and thrombin were removed by fractionation in a Superdex-200 HPLC sizing column (Pharmacia Biotech) as described previously (Enenkel et al., 1995). Alternatively, GST was removed by incubation with glutathione–agarose beads, and thrombin was neutralized by addition of a 1.5 M excess of hirudin (Sigma). Aliquots of purified Kap60 and Kap95 were frozen in liquid nitrogen and stored at −70°C.

Preparation of Recombinant Transport Substrate

The NLS of the large T antigen (TPPKKKRKVEDP) (Kalderon et al., 1984) was used as a template to generate oligonucleotides (5′-GA TCC ACC CCG CCG AAA AAA AAA CGC AAA GTG GAA GAT CCG G-3′ and 5′-AA TTC CGG ATC TTC CAC TTT GCG TTT TTT TTT CGG CGG GGT G-3′) that encode this sequence and that can be ligated directly into the BamHI and EcoRI endonuclease restriction sites of vector pGEX–2TK (Pharmacia Biotech) to create a C-terminal fusion to GST. Likewise, the mutant NLS sequence (TPPKTKRKVEDP) and the inverse NLS sequence (PDEVKRKKKPPT) were used as a template to generate oligonucleotides (5′-GA TCC ACC CCG CCG AAA ACC AAA CGC AAA GTG GAA GAT CCG G-3′ and 5′-AA TTC CGG ATC TTC CAC TTT GCG TTT GGT TTT CGG CGG GGT G-3′; 5′-GA TCC CCG GAT GAA GTG AAA CGC AAA AAA AAA CCG CCG ACC G-3′ and 5′-AA TTC GGT CGG CGG TTT TTT TTT GCG TTT CAC TTC ATC CGG G-3′) that encode these sequences, respectively, and that can be ligated directly into the BamHI and EcoRI endonuclease restriction sites of vector pGEX–2TK (Pharmacia Biotech) as before. Plasmids were introduced separately into the E. coli strain BLR, and soluble fusion proteins in E. coli lysates were purified on glutathione–agarose beads as described for the nucleoporins (see below). The purified GST-NLS chimeras were dialyzed against binding buffer without blocking agents, aliquoted, frozen in liquid nitrogen, and stored at −70°C.

Preparation of Recombinant Nucleoporin FXFG and GLFG Repeat Proteins

To generate recombinant proteins that contain an FXFG repeat region, portions of Nup1 (Davis and Fink, 1990) and Nup2 (Loeb et al., 1993) were expressed as GST fusions. The portion of NUP1 that encodes fifteen consecutive FXFG peptide motifs (amino acids 432–816) was amplified from yeast genomic DNA by PCR using synthetic oligonucleotides that incorporate a BamHI endonuclease restriction site in frame with codon 432 and a stop codon after codon 816 followed by an EcoRI site (see Belanger et al., 1994). Likewise, the portion of NUP2 that encodes sixteen consecutive FXFG peptide motifs (amino acids 186–561) was amplified from yeast genomic DNA by PCR using synthetic oligonucleotides (5′-CCG GGA TCC GAT TCC GTT TTC TCA TTT GGC CCA AAA AAA-3′ and 5′-CCG GAA TTC CTA ACT ACC TTT TTG TTC AAA TGG CAA AGA AAA-3′) that incorporate a BamHI endonuclease restriction site in frame with codon 186 and a stop codon after codon 561 followed by an EcoRI site. The digested PCR products were ligated into vector pGEX–2TK, and the resulting plasmids were transformed into the E. coli strain BLR.

To generate recombinant proteins that contain a GLFG repeat region, portions of Nup145 (Wente and Blobel, 1994; Fabre et al., 1994) and Nup57 (Grandi et al., 1995) were expressed as GST fusion proteins. The portion of the NUP145 that encodes twelve consecutive GLFG peptide motifs (amino acids 20–218) was amplified from yeast genomic DNA by PCR using synthetic oligonucleotides (5′-CGC GGA TCC CCG ACA TCC ACT CCG GCA CAG CCT-3′ and 5′-CCG GAA TTC GGA TCT TGG GAA TGA AGC ACC ATT ATT TCC-3′) that incorporate a BamHI site in frame with codon 20 and an EcoRI site after codon 218. Likewise, the portion of the NUP57 that encodes nine consecutive GLFG peptide motifs (amino acids 72–244) was amplified from yeast genomic DNA by PCR using synthetic oligonucleotides (5′-T CCC CCG GGG AGT ACA GGT GGA GGC CTT TTC GGT AAT-3′ and 5′-CCG GAA TTC CTA CGC AGT GTT TGT TTG AGG CTG CTG GGA-3′) that incorporate a SmaI site in frame with codon 20 and a stop codon followed by an EcoRI site after codon 218. The digested PCR products were ligated into vector pGEX–2TK and transformed into BLR.

Soluble fusion proteins were purified from E. coli lysates as follows. Cells were grown in 350 ml of 2× YTA (Difco Laboratories) at 26°C to a cell density of 2 OD_{600} units. IPTG was added to a final concentration of 0.2 mM, and after 1 hr at 26°C, the cells were harvested at 4°C by centrifugation. Cell pellets were washed once by resuspension in 200 ml of chilled buffer A (20 mM HEPES–KOH [pH 7.4], 1 mM PMSF, 1 mM EGTA) and sedimentation. Cell pellets were resuspended with 40 ml of chilled buffer B (50 mM HEPES–KOH [pH 7.5], 150 mM KOAc, 2 mM DTT, 1 mM PMSF, 2 μg/ml aprotinin, 2 μg/ml pepstatin, 2 mM EGTA, 1 mM EDTA), and cells were lysed using a French pressure cell (900 ψ) (SLM Instruments Incorporated). Cell debris was removed by centrifugation at 20,000 × g for 10 min at 4°C, and the supernatant was filtered through a 0.45 μm syringe filter (Schleicher and Schuell). Portions of the lysate were aliquoted, frozen in liquid nitrogen, and stored at −80°C. To purify the fusion proteins, the filtrate was mixed with 0.5 ml of packed glutathione–agarose beads that were equilibrated in buffer C (20 mM HEPES–KOH [pH 7.4], 150 mM KOAc, 2 mM $Mg(OAc)_2$, 2 mM DTT), and the mix was incubated at 4°C for 1 hr. Beads were collected by centrifugation at 2000 × g for 2 min at 4°C and were washed six times with 15 ml of buffer C by repeated resuspension and centrifugation. To elute the fusion protein, beads were resuspended in 1 ml of buffer C with 10 mM reduced-glutathione and were incubated for 10 min at 4°C. Pooled eluates (3 ml) contained fusion protein at an average concentration of 1 mg/ml. The purified GST chimeras were dialyzed, and a portion of each was subjected to thrombin cleavage for 30 min (1.5 NIH units of thrombin for every 100 μg or more of chimera); this reaction was performed while the chimera was bound to glutathione–agarose beads. At the end of each incubation, a 1.5 M excess of hirudin (Sigma) was added to inhibit further proteolysis, and the glutathione–agarose beads containing GST were removed. Aliquots of cleaved and uncleaved recombinant FXFG and GLFG repeat proteins were frozen in liquid nitrogen and stored at -70°C.

Preparation of Recombinant Ran

Human Ran and its mutant form that can bind but not hydrolyze GTP were isolated as described by Coutavas et al. (1993). Yeast Ran was isolated as follows (a detailed protocol will be published elsewhere; M. Floer and G. B., unpublished data). The *GSP1* gene (Belhumeur et al., 1993) was amplified from yeast genomic DNA using PCR, was inserted into plasmid pET21d (Novagen), and was transformed into the E. coli strain BL21 (DE3) (Novagen). After IPTG induction, cells were lysed using a French pressure cell (900 ψ) (SLM Instruments Inc.). A medium speed supernatant of the cell lysate was obtained, and the concentrations of GTP and $Mg(OAc)_2$ were adjusted to 0.5 mM and 10 mM, respectively. The lysate was applied to a Mono Q anion-exchange column (Pharmacia) and then to a Superdex-75 HPLC sizing column (Pharmacia Biotech). Fractions that contained Ran/Gsp1 were pooled, and aliquots were frozen in liquid nitrogen and stored at −70°C.

When indicated, Ran was preincubated with different nucleotide analogs as follows. Aliquots of Ran (10 μl of a 0.15 μg/ml stock dissolved in binding buffer without blocking agents) were incubated in the presence of 15 mM EDTA, 0.6 mM nucleotide, and 2 mM DTT, for 90 min at room temperature. Magnesium acetate was then added to a final concentration of 30 mM, and the samples were incubated on ice for >15 min. Aliquots were frozen in liquid nitrogen and stored at −80°C.

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References

Adam, E.J.H., and Adam, S.A. (1994). Identification of cytosolic factors required for nuclear location sequence-mediated binding to the nuclear envelope. J. Cell Biol. *125*, 547–555.

Adam, S.A., and Gerace, L. (1991). Cytosolic proteins that specifically bind nuclear location signals are receptors for nuclear import. Cell *66*, 837–847.

Adam, S.A., Sterne-Marr, R.E., and Gerace, L. (1990). Nuclear protein import in permeabilized mammalian cells requires soluble cytoplasmic factors. J. Cell Biol. *111*, 807–816.

Belanger, K.D., Kenna, M.A., Wei, S., and Davis, L.I. (1994). Genetic and physical interactions between Srp1 and nuclear pore complex proteins Nup1p and Nup2p. J. Cell Biol. *126*, 619–630.

Belhumeur, P., Lee, A., Tam, R., DiPaolo, T., Fortin, N., and Clark, M. (1993). Gsp1 and Gsp2, genetic suppressors of the prp20-1 mutant in *Saccharomyces cerevisiae*: GTP binding proteins involved in the maintenance of nuclear organization. Mol. Cell. Biol. *13*, 2152–2161.

Bischoff, R., Krebber, H., Smirnova, E., Dong, W., and Ponstingl, H. (1995). Co-activation of RanGTPase and inhibition of GTP dissociation by Ran-GTP binding protein RanBP1. EMBO J. *14*, 705–715.

Chi, N., Adam, E., and Adam, S. (1995). Sequence and characterization of cytoplasmic nuclear import factor p97. J. Cell Biol. *130*, 265–274.

Coutavas, E., Ren, M., Oppenheim, J., D'Eustachio, P., and Rush, M. (1993). Characterization of proteins that interact with the cell-cycle regulatory protein Ran/TC4. Nature *366*, 585–587.

Davis, L.I., and Fink, G.R. (1990). The *NUP1* gene encodes an essential component of the yeast nuclear pore complex. Cell *61*, 965–978.

Enenkel, C., Blobel, G., and Rexach, M. (1995). Identification of a yeast karyopherin heterodimer that targets import substrate to mammalian nuclear pore complexes. J. Biol. Chem. *270*, 16499–16502.

Fabre, E., Boelens, C., Mattaj, I, and Hurt, E. (1994). Nup145p is required for nuclear export of mRNA and binds homopolymeric RNA in vitro via a novel conserved motif. Cell *78*, 275–289.

Feldherr, C., Kallenbach, E., and Schultz, N. (1984). Movement of a karyophilic protein through the nuclear pores of oocytes. J. Cell Biol. *99*, 2216–2222.

Görlich, D., Prehn, S., Laskey, R., and Hartmann, E. (1994). Isolation of a protein that is essential for the first step of nuclear protein import. Cell *79*, 767–778.

Görlich, D., Kostka, S., Kraft, R., Dingwall, C., Laskey, R., Hartmann, E., and Prehn, S. (1995a). Two different subunits of importin cooperate to recognize nuclear location signals and bind them to the nuclear envelope. Curr. Biol. *5*, 383–392.

Görlich, D., Vogel, F., Mills, A., Hartmann, E., and Laskey, R. (1995b). Distinct functions for the two importin subunits in nuclear protein import. Nature *377*, 246–248.

Grandi, P., Schlaich, N., Tekotte, H., and Hurt, E. (1995). Functional interaction of Nic96p with a core nucleoporin complex consisting of Nsp1p, Nup49p, and a novel nucleoporin Nup57p. EMBO J. *14*, 76–87.

Imamoto, N., Tachibana, T., Matsubae, M., and Yoneda, Y. (1995a). A karyophilic protein forms a complex with cytoplasmic components prior to nuclear pore binding. J. Biol. Chem. *270*, 8559–8565.

Imamoto, N., Shimamoto., T., Takao, T., Tachibana, T., Kose, S., Matsubae, M., Sekimoto, T., Shimonishi, Y., and Yoneda, Y. (1995b). *In vivo* evidence for involvement of a 58 kDa component of nuclear pore-targeting complex in nuclear protein import. EMBO J. *14*, 3617–3626.

Kadowaki, T., Chen, S., Hitomi, M., Jacobs, E., Kumagui, C., Liang, S., Schmiter, R., Singleton, D., Wisniewska, J., and Tartakoff, A. (1994). Isolation and characterization of *Saccharomyces cerevisiae* mRNA transport-defective (mtr) mutants. J. Cell Biol. *126*, 649–659.

Kalderon, D., Robert, B., Richardson, W.D., and Smith, A.E. (1984). A short amino acid sequence able to specify nuclear location. Cell *39*, 499–509.

Kraemer, D., Strambio-de-Castillia, C., Blobel, G., and Rout, M. (1995). The essential yeast nucleoporin NUP159 is located on the cytoplasmic side of the nuclear pore complex and serves in karyopherin-mediated binding of transport substrate. J. Biol. Chem. *270*, 19017–19021.

Loeb, J., Davis, L., and Fink, G. (1993). NUP2, a novel yeast nucleoporin, has functional overlap with other proteins of the nuclear pore complex. Mol. Biol. Cell *4*, 209–222.

Melchior, F.B., Paschal, J., Evans, J., and Gerace, L. (1993). Inhibition of nuclear protein import by nonhydrolyzable analogs of GTP and identification of the small GTPase Ran/TC4 as an essential transport factor. J. Cell Biol. *123*, 1649–1659.

Moore, M.S., and Blobel, G. (1993). The GTP-binding protein Ran/TC4 is required for protein import into the nucleus. Nature *365*, 661–663.

Moore, M.S., and Blobel, G. (1994). Purification of a Ran-interacting protein that is required for protein import into the nucleus. Proc. Natl. Acad. Sci. USA *91*, 10212–10216.

Moroianu, J., Blobel, G., and Radu, A. (1995a). Previously identified protein of uncertain function is karyopherin α and together with karyopherin β docks import substrate at the nuclear pore complex. Proc. Natl. Acad. Sci. USA *92*, 2008–2011.

Moroianu, J., Hijikata, M., Blobel, G., and Radu, A. (1995b). Mammalian karyopherin $\alpha_1\beta$ and $\alpha_2\beta$ heterodimers: α_1 or α_2 bind nuclear localization signal and β interacts with peptide repeat containing nucleoporins. Proc. Natl. Acad. Sci. USA *92*, 6532–6536.

Paschal, B.M., and Gerace, L. (1995). Identification of NTF2, a cytosolic factor for nuclear protein import that interacts with nuclear pore complex protein p62. J. Cell Biol. *129*, 925–937.

Radu, A., Moore, M.S., and Blobel, G. (1995a). The peptide repeat domain of nucleoporin Nup98 functions as a docking site in transport across the nuclear pore complex. Cell *81*, 215–222.

Radu, A., Blobel, G., and Moore, M.S. (1995b). Identification of a protein complex that is required for nuclear protein import and mediates docking of import substrate to distinct nucleoporins. Proc. Natl. Acad. Sci. USA *92*, 1769–1773.

Richardson, W.D., Mills, A.D., Dilworth, R.A., Laskey, R., and Dingwall, C. (1988). Nuclear protein migration involves two steps: rapid

binding at the nuclear envelope followed by slower translocation through nuclear pores. Cell *52*, 655–664.

Rout, M., and Blobel, G. (1993). Isolation of the yeast nuclear pore complex. J. Cell Biol. *123*, 771–783.

Rout, M., and Wente, S. (1994). Pores for thought: nuclear pore complex proteins. Trends Biochem. Sci. *4*, 357–365.

Schlenstedt, G., Saavedra, C., Loeb, J., Cole, C., and Silver, P. (1995). The GTP-bound form of yeast Ran/TC4 homologue blocks nuclear protein import and appearance of poly-$(A)^+$ RNA in the cytoplasm. Proc. Natl. Acad. Sci. USA *92*, 225–229.

Walter, P., and Blobel, G. (1982). Signal recognition particle contains a 7S RNA essential for protein transport across the endoplasmic reticulum. Nature *299*, 691–698.

Weis, K., Mattaj, I., and Lamond, A. (1995). Identification of hSRP1a as a functional receptor for nuclear localization sequences. Science *268*, 1049–1051.

Wente, S., and Blobel, G. (1994). NUP145 encodes a novel yeast glycine–leucine–phenylalanine–glycine (GLFG) nucleoporin required for nuclear envelope structure. J. Cell Biol. *125*, 955–969.

Yano, R., Oakes, M., Yamaghishi, M., and Nomura, M. (1992). Cloning and characterization of *SRP1*, a suppressor of temperature-sensitive RNA polymerase I mutations in *Saccharomyces cerevisiae*. Mol. Cell. Biol. *12*, 5640–5651.