GLFG and FxFG Nucleoporins Bind to Overlapping Sites on Importin-β*

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The interaction between nuclear pore proteins (nucleoporins) and transport factors is crucial for the translocation of macromolecules through nuclear pores. Many nucleoporins contain FG sequence repeats, and previous studies have demonstrated interactions between repeats containing FxFG or GLFG cores and transport factors. The crystal structure of residues 1–442 of importin-β bound to a GLFG peptide indicates that this repeat core binds to the same primary site as FxFG cores. Importin-β-1178D shows reduced binding to both FxFG and GLFG repeats, consistent with both binding to an overlapping site in the hydrophobic groove between the A-helices of HEAT repeats 5 and 6. Moreover, FxFG repeats can displace importin-β or its S. cerevisiae homologue, Kap95, bound to GLFG repeats. Addition of soluble GLFG repeats decreases the rate of nuclear protein import in digitonin-permeabilized HeLa cells, indicating that this interaction has a role in the translocation of carrier-cargo complexes through nuclear pores. The binding of GLFG and FxFG repeats to overlapping sites on importin-β indicates that functional differences between different repeats probably arise from differences in their spatial organization.

The bidirectional exchange of macromolecules between the nucleus and cytoplasm takes place through nuclear pore complexes (NPCs) that perforate the nuclear envelope (reviewed by Refs. 1–4). The active transport of large macromolecules through NPCs requires escort or carrier proteins that shuttle rapidly between the two cellular compartments. Members of the importin-β/karyopherin-β family function as carriers for many nuclear trafficking processes including nuclear protein import and export and tRNA export (reviewed by Refs. 2 and 3). However, not all nuclear trafficking is mediated by members of this family. For example, the NXF family is important for mRNA export (reviewed by Refs. 5 and 6), and NTF2 mediates nuclear import of RanGDP (7, 8).

Transport by carrier proteins is thought to be a sequential stepwise process. For example, importin-β binds cargo molecules in the cytoplasm, either directly or via an adapter such as importin-α, the complex moves through the NPC, and then cargo is released in the nucleus on importin-β binding RanGTP. The importin-β-RanGTP complex is recycled to the cytoplasm where it is dissociated by hydrolysis of the GTP on Ran, freeing importin-β for another round of import (reviewed by Refs. 9 and 10). Although the actual mechanism of translocation through NPCs remains controversial, a shared tenet of each model involves binding of carrier molecules to a subset of NPC proteins (termed nucleoporins or “Nups”) that contain domains with repeating Phe-Gly (FG) sequence motifs (Refs. 11–14, reviewed in Refs. 15 and 16). These repeat have hydrophobic cores based on FG, GLFG, or FxFG motifs separated by hydrophilic linkers of variable sequence and length. In vitro, FG-Nups interact directly with a broad range of transport factors and there is compelling in vivo evidence that interaction between carrier and FG-Nups is important for at least one stage of translocation through NPCs (Ref. 11, and reviewed by Refs. 16 and 17). FG-Nups may function to concentrate carrier-cargo complexes at the NPC entrance (18) or participate in a sequence of docking and undocking interactions as the carrier-cargo complexes transit through NPCs (Refs. 11, 13, and 14, reviewed by Ref. 16). Alternatively, FG-Nups may form a meshwork within the central channel that is permeable only to molecules that interact with FG repeats (19).

Rout and Wente (15) suggested that FG-Nups may divide into different subfamilies, based on differences in the primary core sequences between different Nups (FG, GLFG, and FxFG) and on differences in linker sequences. In addition, the distribution of different FG-Nup subfamilies within NPCs is not the same (18, 20). The most complete analysis has been possible in Saccharomyces cerevisiae where there are 13 different FG-Nups. The GLFG-Nups are found on both sides of the NPC, some FG-Nups are exclusively on the cytoplasmic side, and some FxFG-Nups are exclusively on the nuclear side (18). Individual FG-Nups show distinct binding preferences for different carriers (11), suggesting that potentially some may have specialized functions. This possibility is also supported by a range of genetic, biochemical, and in vivo evidence implicating different FG-Nups in import or export events (Refs. 1 and 21–24, reviewed in Refs. 11, 17, 25, and 26).

X-ray crystallographic studies (Refs. 27–29, reviewed in Ref. 30) have shown that importin-β is a helicoidal molecule constructed from 19 HEAT repeats, each formed by a pair of α-helices (the A- and B-helices). A combination of biochemical...
Importin-β/FGN nucleoporin Interaction

and structural studies have indicated that, although FxFG-Nups may bind at several sites on importin-β, the primary site is located in the N-terminal half of the molecule between the A-helices of HEAT repeats 5 and 6 (21, 31, 32). However, fragments containing the C-terminal half of importin-β do not show such binding to nuclear envelopes (31, 32). The crystal structure of residues 1–442 of importin-β complexed with FxFG repeats (21) shows two putative FxFG sites, but the site between the A-helices of repeats 5 and 6 has higher occupancy. Moreover, mutations at this site (for example, I178D) weaken the binding of FxFG constructs considerably, consistent with its representing the primary FxFG binding site (21). The binding of importin-β to FG-Nups as well as to cargo and/or importin-α is disrupted by RanGTP (11, 14, 33–35) and comparison of different co-crystal structures suggests that RanGTP binding may induce a conformational change in importin-β that occludes the primary FxFG binding site (21). It has not been established unequivocally if such a mechanism contributes to vectorial movement through NPCs or release of importin-β from Nups.

Crystal structures of FxFG repeat cores bound to importin-β and NTPF2 (21, 36) and an FG core bound to the mRNA export factor Tap/NSP1 (37) indicate that, in each complex, the interaction is dominated by repeat core Phe side chains being buried in a hydrophobic pocket. Mutations in this pocket reduced the binding of these transport factors to FG-Nups (21, 36, 37). In addition, the affinity of FxFG repeat peptides for the UBA domain of Tap was dramatically reduced when the second Phe, F2, was mutated to Ala (38). Overall, these data indicate that the hydrophobic cores of FG repeats are crucial to their binding to transport factors, although they do not rule out there also being contributions from the linkers.

To determine the precise function of each interaction in a cycle of productive nuclear transport, it is crucial to define how the FG repeats are recognized by nuclear transport factors. To address this question, we have investigated the interaction of GLFG- and FxFG-Nups with importin-β, and report here the 2.8 Å resolution crystal structure of a complex between residues 1–442 of importin-β and a GLFG repeat containing peptide. The GLFG core binds to the same hydrophobic site on importin-β previously identified as the principal FxFG binding site (21). I178D-importin-β, which shows reduced affinity for FxFG repeats, also shows reduced binding to GLFG repeats and nuclear protein import is inhibited by soluble GLFG repeats. Moreover, we show that the binding of GLFG repeats to importin-β is inhibited by soluble FxFG repeats confirming that, in solution, the GLFG and FxFG repeat cores bind primarily to overlapping sites on importin-β. Similar competition between GLFG- and FxFG-Nups was observed with Kap95.

MATERIALS AND METHODS

Protein Preparation—The cloning, expression, and purification of human importin-β and its I178D mutant, Ib442, canine Ran, and the S. cerevisiae Nsp1 FF18 (containing eighteen FxFG repeats) have been previously described (21, 39, 40). GST-tagged constructs of Nup100 residues 2–610 (41), Nup116 residues 161–730 (22), and the FxFG repeat containing domain of Nup1 residues 423–816 were purified on glutathione-Sepharose 4B as described by the suppliers (Amersham Biosciences). GLFG peptide (DSGGGLFGSK) was dissolved in Milli Q water buffered to pH 7.4 with Tris (2–20 mM).

Crystalization and Data Collection—Crystals of the Ib442-GLFG complex were obtained by vapor diffusion using hanging drops of 6 µl composed of 3 µl of well buffer and 3 µl of Ib442/GLFG mixture (15 mg/ml Ib442, 25 mM GLFG peptide). Well buffer was composed of 100 mM ammonium acetate, pH 6.0, and 1.2 mM ammonium sulfate. Crystals were slowly dehydrated by transfer to a hanging drop containing 2.8 M ammonium sulfate, 100 mM ammonium acetate, pH 6.0, 5 mM GLFG peptide above a well containing 3.2 mM ammonium sulfate, 100 mM ammonium acetate, pH 6.0. After 2 days the crystals were transferred to 3.2 mM ammonium sulfate, 5% (v/v) glycerol, 100 mM ammonium acetate, pH 6.0, before plunge-freezing into liquid nitrogen. Ib442/GLFG crystals vitrified in this way diffracted to 2.8 Å resolution using 1.488 Å wavelength radiation and a Mar345 detector on beamline 14.1 at the SR (Daresbury, UK). Data were integrated using MOSFLM (42) and reduced using SCALA (42).

Crystals of Ib442-FF5 with P2₁,2,2₁, symmetry were grown under similar conditions to the P2₁,2,2 crystals (21) by vapor diffusion using 1.16 M hanging drop composed of 3 µl of drop buffer, 3 µl of Ib442 protein (4.5 mg/ml stock solution), and 1 µl of FF5 (11 mg/ml stock solution). Reservoir buffer was 1.2–1.28 M ammonium sulfate, 100 mM ammonium acetate, pH 5.9, 30 mM DTT and drop buffer was 1.16–1.2 M ammonium sulfate, 200 mM ammonium acetate, 25 mM DTT. Diffraction-quality crystals (dimensions 200 × 50 × 50 µm), which diffracted past 3 Å, were transferred to a drop containing 24% glycerol for less than 1 min and flash-frozen at 100 K. A native data set was collected at 100 K using 0.934 Å wavelength radiation on beamline ID14-EH1 at ESRF (Grenoble, France) using a MarCCD detector.

Structure Solution—Molecular replacement and refinement used the CNS package (43). The structures of the crystals of Ib442 complexed to either GLFG or FxFG peptides were determined by molecular replacement using residues 1–442 of importin-β bound to the IBB domain (28) as a model. Although the coordinates of Ib442 when bound to FF5, in the original Ib442-FF5 crystal form, was a more similar starting model than the IBB-bound structure, we thought it prudent to avoid the possibility of introducing bias arising from a Nup-bound Ib442 as a model. The Ib442 chains were rebuilt locally and refined using simulated annealing before examining Fₚ₋ₚ for difference maps to locate putative bound Nup repeats. In both cases, characteristic tube-like or ribbon-like density structures could be observed from which there were bulky protrusions that could be identified as Phe and Leu side chains. Structural figures were made using MOLSCRIPT (44), BOBSCRIPT (45), and Raster3D (46).

Solution Binding Assays—Saturating amounts of GST-Nup were incubated with 200 µl of packed glutathione-Sepharose beads (Amersham Biosciences) for 1 h in binding buffer (phosphate-buffered saline (PBS) supplemented with 0.05‰ Tween 20, 2 mM MgCl₂, and 1 mM EDTA) and incubated with 1 ng of Ib442 or GST per well for 16 h at 4 °C on a rotator. After incubation, the beads were washed three times with 1 ml of binding buffer, resuspended in sample buffer (47), and analyzed by SDS-PAGE.

Microtiter Plate Binding Assay—Solid phase binding assays were carried out on Immulon II microtiter plates (Dynex) essentially as described by Ben-Efraim and Gerace (34). Plates were coated with 25 ng Nip FF18, GST-Nup100 (residues 2–610), GST-Nup116 (residues 161–730), or GST per well for 40 min at 4 °C in coating buffer (PBS supplemented with 2 mM DTT and 0.2 mM phenylmethylsulfonyl fluoride). After adsorption the plates were washed three times with PBS and incubated overnight at 4 °C in binding buffer (coating buffer supplemented with 3% BSA and 0.1% Tween 20). Binding reactions were carried out for 2 h at 4 °C with 100 µl/well of the indicated amounts of S-tagged importin-β (wild-type or I178D mutant) or Kap95 proteins in binding buffer. After binding, plates were washed three times by immersion in binding buffer without BSA and proteins were cross-linked for 15 min at room temperature by incubation in 1 mg/ml 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (Pierce) in the same buffer. The wells were then washed for 20 min in PBS-T (PBS supplemented with 0.2% Tween 20), 10 min with PBS-T containing 100 µM Nε-l-lysine and 10 min with PBS-T containing 200 µM Nε-l-lysine. The bound material was removed by washing with binding buffer. For each binding reaction, 40 µl of bead slurry (50% suspension) in 1 ml of binding buffer was incubated with 10 µg of wild type or I178D importin-β for 40 min at 4 °C. For competition assays, 100 µg of FF18 or 60 µg of importin-α were included in the incubation. After incubation, beads were washed twice with 1 ml of binding buffer, resuspended in sample buffer (47), and analyzed by SDS-PAGE.

Yeast Manipulations—Kap95, importin-β, and I178D-importin-β were fused in-frame behind LexA in pCH342 (gift of C. Hardy, Vanderbilt University Medical Center, Nashville, TN) to generate pSW1381, pSW1371, and pSW1449, respectively. The plasmids were transformed...
TABLE I

Crystallographic data for Ib442/Nup crystals

| Crystallographic data for Ib442/Nup crystals |
|---------------------------------------------|
| Complexed with GLFG peptide | Complexed with FF5ex |
| **Data collection** | | |
| Resolution range (Å) | 67.4–2.8 (2.95–2.8)b | 60.9–2.8 (2.95–2.8)b |
| Observations/unique reflections | 257406/48209 | 565208/53288 |
| Completeness (%) | 91.8 (89.6)b | 94.7 (88.8)b |
| Multiplicity | 2.6 (2.3)b | 3.9 (3.4)b |
| Rmerge (I) | 0.062 (0.314)b | 0.092 (0.387)b |
| Rfactor (I) | 7.9 (2.4)b | 5.5 (1.7)b |
| **Refinement** | | |
| Resolution range (Å) | 20–2.8 | 20–2.9 |
| Residues (waters) | 880 (30) | 1350 (13) |
| R-factor (Rmerge) | 0.238 (0.269) | 0.236 (0.273) |
| Bond length root mean square (Å) | 0.013 | 0.018 |
| Bond angle root mean square (degrees) | 1.4 | 1.4 |
| Ramachandran plot (%) | | |
| Most favored | 90.1 | 89.1 |
| Allowed | 9.9 | 10.4 |
| Generously allowed | 0 | 0.5 |
| Forbidden | 0 | 0 |

a FF5 is a construct from S. cerevisiae Nsp1p that contains five FxFG repeats (21).
b The highest resolution shell is in parentheses.

d The highest resolution shell is in parentheses.

RESULTS

Crystal Structure of Ib442 Bound to a GLFG Peptide—We investigated the structural basis for the interaction between importin-β and GLFG repeats using crystals of importin-β residues 1–442 (Ib442) complexed with a peptide of the sequence DSGGGLFGSK. By analogy with the binding to FXG repeats (50), we anticipated that the binding of GLFG repeats to importin-β would probably be mediated largely by their hydrophobic cores, and so we employed ammonium sulfate as the precipitant. In this way we obtained crystalline plates, measuring 0.1 × 0.05 × 0.02 mm, which had P2₁ symmetry and diffracted past 2.8-Å resolution (Table I) using synchrotron radiation. We also obtained crystals of putative Ib442-GLFG complexes using several repeats derived from Nup116, but none diffracted to high resolution.

Molecular replacement identified only two Ib442 chains in the asymmetric unit. Although this gave an unusually high (77.3%) solvent content (51), rigid body refinement gave an R-factor of 37.1% that was reduced to 29.3% (Rmerge, 31.7%) after simulated annealing and local model rebuilding. These statistics indicated that it was extremely unlikely that there was an additional Ib442 chain present and there was no Fc – Fc electron density indicative of a third Ib442 chain. The crystal lattice contained large square solvent channels formed by the intersection of approximately perpendicularly Ib442 chains. This configuration exposed a large proportion of the surface of each chain to solvent and, by extension, to the GLFG peptide. After refinement, both 2Fo – Fc and Fo – Fc a-weighted electron density maps showed tubes of additional density associated with the Ib442 chains in two different positions that could be modeled as GLFG peptide cores (Fig. 1A). Including these peptides reduced the R-factor to 23.6% (Rmerge, 27.3%) after refinement and rebuilding. Overall, the final structure had good stereochemistry (Table I).

One putative GLFG repeat core was located between the A-helices of HEAT repeats 5 and 6 (site 1, Figs. 1C and 2A) at the same location as the primary FxFG binding site observed in Ib442-FF5 (21). A second GLFG core was bound at a crystal contact between two Ib442 chains (site 2, Figs. 1C, Fig. 2, C and F). The GLFG peptide at the crystal packing interface contacted residues in HEAT repeats 2 and 3 from one chain and repeats 6 and 7 from the other. However, the site 2 binding pocket in which the hydrophobic core was buried was formed partially from each Ib442 chain and neither individually buried the hydrophobic Phe and Leu side chains effectively (Fig. 2, C and F). A similar conformation was adopted by the GLFG peptide at both importin-β binding sites, and in each case the interaction was primarily hydrophobic. The buried surface area (Table II) and extensive molecular contacts between the GLFG core and the two Ib442 molecules at the crystal packing inter-
face (site 2) were comparable with those at site 1. However, because the GLFG peptide at site 2 interacts with two importin-β chains, it is unlikely to contribute to nucleoporin binding in solution to the same extent as the primary site. Indeed, the surface buried in the interface between the GLFG core at site 2 and either of the two individual importin-β chains was substantially smaller (Table II). Moreover, the Leu side chain of the FxFG core interacted with one importin chain while the Phe of the core interacted with the other. Thus, the interaction observed at site 2 would only be of physiological significance in the context of an importin-β dimer. Importin-β itself has not been reported to dimerize and so we concluded that the interaction observed at site 2 was most probably a crystallization artifact. In contrast, site 1 is not located near a crystal contact, and, as demonstrated by several lines of evidence presented below, constitutes a bona fide binding site for GLFG repeats on importin-β.

Comparison between the Binding of GLFG and FxFG Repeat Cores to Ib442—Although our original P2,2,2 Ib442-FxFG crystals (21) were adequate to identify both the location of the FxFG binding site on importin-β and the key residues involved in the interaction, their diffraction was highly anisotropic. Although data extended past 2.8 Å along one axis, diffraction was weak past 3.4 Å along the others. To enable a more precise comparison to be made of the binding of FxFG and GLFG cores, we obtained a second Ib442-FxFG crystal form that yielded higher quality diffraction data (Table I). These crystals had P21212 symmetry and produced a complete 2.8-Å resolution data. They were solved by molecular replacement to give an R-factor of 23.6% (Rfree 26.7%) after refinement and contained three Ib442 chains in the asymmetric unit, each of which bound a FxFG core essentially as observed in the original P2,2,2 crystals (Fig. 2, B and E).

The GLFG peptide at site 1 followed a path similar to that adopted by FxFG repeats bound to the same site (Ref. 21 and Fig. 2G). Thus, the Phe-Gly moiety of the GLFG core adopted a position within the binding site closely matching the analogous FG in the FxFG peptide. As observed in the FxFG construct (Ref. 21 and see below), the aromatic Phe residue was inserted into a hydrophobic cavity formed by the side chains of residues Leu174, Thr176, Ile178, Glu214, Phe217, and Ile218 of importin-β (Fig. 2, A and B). The GLFG core adopted a different backbone conformation from the FxFG core (Fig. 2). Whereas the FxFG core adopted a β-conformation, with its two Phe rings pointing into the groove between the A-helices of HEAT repeats 5 and 6 of importin-β and the “X” residue (here Ser) pointing away from the surface, the GLFG core formed a loop with both central residues (LF) of the GLFG repeat pointing into the groove between HEAT repeats 5 and 6. The Leu side chain of the repeat followed a path similar to the peptide backbone of the FxFG cores (Fig. 2G). Overall, the different conformations adopted by the two repeat types produced for each the most efficient burying of their hydrophobic residues into the binding site on importin-β.

The interaction of GLFG repeats with the primary site of importin-β buried slightly less surface area than was buried by the FxFG interaction (Table II). The FxFG cores appeared to fit to the importin-β primary site more effectively than GLFG, as shown, for example, in Fig. 2, where the first Phe, F1, of the FxFG core clearly occupies more of the volume of the site than the Leu of the GLFG. Moreover, the Leu side chain is less involved in molecular contacts with importin-β, whereas in FxFG repeats both Phe are in contact. Also, because of the different backbone conformation of GLFG repeats, the core is only able to form one of the two putative H-bonds observed for FxFG cores. The GLFG peptide was positioned so that it could form a putative H-bond with Glu214, but it was not positioned close enough to Thr176 to form a H-bond with it as well (Fig. 2).

In summary, although the binding site on importin-β was able to recognize both classes of Nup core, it formed a more intimate contact with those from FxFG-Nups.

Of the two large hydrophobic residues in each FG repeat, the side chain of the second (i.e. the Phe of the FG-dipeptide) formed a more intimate contact with the surface of importin-β than the side chain of the first (i.e. the Leu of the GLFG repeat, or F1 of the FxFG repeat). Thus, whereas the side chain of the second Phe, F2, is wholly buried in a hydrophobic pocket, the Leu/F1 side chains were more exposed to solvent. Thus the

Fig. 1. Binding of GLFG and FxFG repeats to Ib442. Annealed omit F – F, maps contoured at 2.5σ at the primary GLFG binding site (A) (site 1, see C) and the primary FxFG binding site (B). C, the GLFG peptide showed difference density located at two sites on Ib442. Site 1 (black) was located between the A-helices of HEAT repeats 5 and 6 and was also the primary site at which FxFG cores bound, whereas site 2 (red) was located at a contact between two Ib442 chains in the crystal and was thought to be a crystallization artifact.
molecular recognition of FG repeats by importin-β appears to involve primarily the FG-dipeptide. Although a contribution to the interface is also made by “Leu/F1,” which interacts with hydrophobic components of the binding site, its primary function appears to be to form a cap or seal over F2 that further shields it from solvent. This capping feature was observed in both FxFG and GLFG complexes and may be a general feature of FG repeat binding to importin-β. The Pro of a PSFG repeat (see, for example, Ref. 1) may perform a similar function.

Although the crystal data indicate that both FxFG and GLFG repeat cores bind in an analogous way to the hydrophobic groove formed between the A-helices of importin-β HEAT repeats 5 and 6, these results were obtained with an importin-β fragment and a peptide with a single GLFG sequence. To ensure that these results reflected genuine interaction sites, we conducted biochemical binding studies using full-length importin-β or Kap95 and domains from GLFG and FxFG nucleoporins that contain large numbers of repeats (see below).

**FIG. 2. Details of the interaction between GLFG and FxFG cores and Ib442.** Stereo views of GLFG (A, black) and FxFG (B, red) cores show that each binds in the hydrophobic pocket (yellow) formed between HEAT repeats 5 (light green) and 6 (light blue) by the side chains of Leu174, Thr175, Ile178, Glu214, Phe217, and Ile218. In addition to the hydrophobic interaction, putative H-bonds are formed between the main chain and Glu214. Thr175 forms a H-bond to the FxFG core but not to the GLFG core. Surface views (D and E) illustrate the intimacy of the contact between the repeat cores and Ib442. In contrast, the contact between the GLFG peptide at site 2 involved a pocket formed by two Ib442 chains (green and orange in C and F) and neither Ib442 chain alone buried a significant amount of the core, consistent with site 2 representing a crystallization artifact and site 1 representing the true GLFG binding site. G, although the conformation of GLFG and FxFG cores bound to Ib442 was different, they had a similar outline.
I178D-Importin-β Binds Less Strongly to Both FxFG and GLFG Repeats—Previous work (21) indicated that Ile\textsubscript{178}, located between the A-helices of HEAT repeats 5 and 6 (Fig. 2), was a crucial component of the primary FxFG binding site on importin-β and showed that the I178D mutant reduced the binding of FxFG repeats to below detectable levels. This mutant retained wild type affinity for RanGTP and importin-α (mutant/wild-type \( K_d \) values measured in microtiter plate binding assays (34) were 8.9 and 5.8 nM, respectively (data not shown)) indicating that it had not introduced a major, global conformational change in importin-β. To test whether the mutant also perturbed interactions with GLFG repeats, binding experiments were conducted. Initially, bacterially expressed GST fusions containing the GLFG regions of Nup100 (residues 2–610) and Nup116 (residues 161–730) were coupled to glutathione-Sepharose 4B (Amersham Biosciences), washed and incubated with 10
\( \mu \)g of wild type (lanes 1, 5, and 9) or I178D (lanes 3, 7, and 11) importin-β. Bound proteins were eluted with Laemmli sample buffer and analyzed by SDS-PAGE. Binding of wild type importin-β to GST-Nups was also done in the presence of 100
\( \mu \)g of soluble Nsp1 FF18 (residues 262–603, lanes 2, 6, and 10) or 60
\( \mu \)g of importin-α (lanes 4, 8, and 12).

**Table II**

| Buried surface area/ Å\(^2\) | Site 1 | Site 2 |
|-----------------------------|--------|--------|
| Buried surface area         |        |        |
| Site 1                      | FxFG   | 593 (673)* |
| GLFG                        | 534    |         |
| Site 2                      | Both chains | 556 |
| Single chains               | 334, 296 |       |

*Figure in parentheses is for pentapeptide AFSFG, lower value is for FSFG.

**Fig. 3.** Importin-β binds both FxFG and GLFG nucleoporins at overlapping sites. GST-Nups containing FxFG repeats (Nup1 residues 423–816, lanes 1–4) or GLFG repeats (Nup100 residues 2–610, lanes 5–8, and Nup116 residues 161–730, lanes 9–12) were coupled to glutathione-Sepharose 4B (Amersham Biosciences), washed and incubated with 10
\( \mu \)g of wild type (lanes 1, 5, and 9) or I178D (lanes 3, 7, and 11) importin-β. Bound proteins were eluted with Laemmli sample buffer and analyzed by SDS-PAGE. Binding of wild type importin-β to GST-Nups was also done in the presence of 100 \( \mu \)g of soluble Nsp1 FF18 (residues 262–603, lanes 2, 6, and 10) or 60
\( \mu \)g of importin-α (lanes 4, 8, and 12).

**Fig. 4.** Importin-β and Kap95 bind both FxFG and GLFG nucleoporins. The affinity of importin-β and Kap95 for FxFG and GLFG constructs was measured using solid phase binding assays (34). Binding of S-tagged importin-β (○) or I178D-importin-β (△) to Nsp1 FF18 (A) (residues 262–603) or GST-Nup116 (B) (residues 161–730). Binding of S-tagged Kap95 (△) to Nsp1-FF18 (C) or GST-Nup116 (D) (residues 161–730). Lineweaver-Burk plots for importin-β/Nsp1 FF18 (E), importin-β/Nup116 (F), Kap95/Nsp1 FF18 (G), and Kap95/Nup116 (H). These binding isotherms were used to calculate the apparent dissociation constants (see Table III).
under conditions where the importin-β/FxFG repeat interaction was observed (24). Nonetheless, the binding of importin-β to GLFG repeats is stronger than that reported for the NTF2/FxFG repeat interaction (1.2 µM; see Refs. 52 and 53) that is essential for NTF2 to import Ran into the nucleus. Therefore, the interaction between importin-β and GLFG repeats is within the range expected for a role in nuclear trafficking. In agreement with the pull-down assays (Fig. 3), importin-β I178D bound much more weakly to either FxFG or GLFG repeats compared with wild-type importin-β (Fig. 4, A and B, Table III). The strength of the interaction between importin-β and Nups is severely reduced by RanGTP (11, 14, 34). Moreover, we observed that binding of importin-β to both Nsp1-FF18 and GST-Nup100(2–610) was inhibited by RanGTP (but not RanGDP), thus demonstrating the specificity of the interaction (data not shown).

GLFG and FxFG Repeats Compete for Overlapping Sites on Importin-β and Kap95—Cross-competition studies were conducted between FxFG and GLFG-containing Nups for binding importin-β in both pull-down assays (Fig. 3) and solid-phase assays (Fig. 5A). FxFG repeats from Nsp1 displaced wild-type importin-β bound to either GST-Nup1 (FxFG) or GST-GLFG recombinant protein derived from Nup100 or Nup116 (Fig. 3, lanes 2, 6, and 10, respectively; Fig. 5A). This competition between different repeat types confirms that the FxFG and GLFG repeats bind to overlapping sites on importin-β.

Because our binding studies used a vertebrate transport factor with GLFG and FxFG repeat domains derived from S. cerevisiae proteins, we also investigated the extent to which these interactions might be influenced by the heterologous origin of the proteins employed. In one set of experiments, we tested whether the S. cerevisiae importin-β homologue, Kap95, bound FxFG and GLFG repeats in a similar manner as importin-β. As shown in Fig. 4 and Table III, S-tagged Kap95 bound the FxFG repeats of Nsp1 and Nup1 and also the GLFG repeats of Nup100 and Nup116 in a comparable manner to importin-β, albeit Kap95 showed a marginally greater affinity for GLFG- than FxFG-containing proteins. Significantly, Nsp1-FF18 was able to displace Kap95 bound to immobilized Nup116 (Fig. 5A), consistent with Kap95, like importin-β, binding both FxFG- and GLFG-Nups at common sites. In addition, we found that Nup100 could displace importin-β or Kap95 bound to Nup116 (Fig. 5B). These results are entirely consistent with those obtained using vertebrate importin-β, indicating that the binding of both types of repeat to common sites is a general feature of at least this subset of nuclear transport factors rather than being species-specific.

We also tried to use the vertebrate GLFG-nucleoporin Nup98 (13, 23) to probe interactions with importin-β, because both we (21) and others (for example, Refs. 13 and 54) had observed that this protein bound importin-β in blot overlays. However, other studies had either failed to detect an interaction between Nup98 and importin-β (24) or had indicated that the interaction was nonspecific and nonsaturable (34). When we investigated this binding more thoroughly, we too found that the binding of importin-β to both native Nup98 from rat liver nuclear envelopes and bacterially expressed GST-Nup98 (residues 43–518) was nonspecific. For example, Nup98 bound S-protein-horseradish peroxidase just as effectively in the absence of S-tagged-importin-β, and in solid-phase assays, importin-β failed to show saturable binding to the bacterially expressed GLFG-containing domain of Nup98 (data not shown). We also attempted to use the yeast two-hybrid system to investigate interactions between Nup98 and either importin-β or Kap95, but the expression levels of Nup98 were low, and we were not able to detect any interaction in this way (data not shown).
shown). Consequently, it was not possible to assay the binding of Nup98 to importin-β.

The binding of FxFG and GLFG repeats to overlapping sites on Kap95 appeared inconsistent with previous studies analyzing a mutant Kap95-L63A (22). In the two-hybrid assay, Kap95-L63A failed to interact with the GLFG regions of Nup116 and Nup100 but retained interaction with the Nup1-FxFG repeat region. To try to resolve this issue, we examined the binding of Kap95-L63A to FxFG and GLFG repeat regions using solution pull-down and solid phase binding assays. Bacterially expressed Kap95-L63A bound both GST-Nup116-GLFG and Nsp1 FF18 in vitro at 4°C (data not shown). Furthermore, the apparent dissociation constants for the binding of Kap95-L63A to both types of repeat regions were similar to wild-type Kap95 under these assay conditions. Therefore, the L63A mutant likely indirectly perturbs nucleoporin binding in the context of the two-hybrid assay. This may be a reflection of the RanGTP environment in the nucleus wherein the two-hybrid interactions are measured and will require future analysis.

Importin-β Requires FG Nup Binding for Function in Yeast—To examine whether the similar FG binding interactions for Kap95 and importin-β reflected identical functional properties, we tested if expression of importin-β could complement the lethal phenotype of a kap95 null yeast strain. Plasmids expressing either wild-type LexA-importin-β or I178D mutant were transformed into a kap95 null tester strain harboring a URA3 plasmid. Interestingly, we found that LexA-I178D-importin-β, LexA-I178D-importin-β, or LexA-KAP95, Complementation was assayed on synthetic complete media containing 2% glucose and 5-fluoroorotic acid at 23 °C for 7 days. Expression of the LexA constructs and the absence of Kap95 in cells expressing LexA-importin-β were confirmed by immunoblot.

Fig. 6. LexA-importin-β, but not LexA-I178D-importin-β, is able to complement kap95Δ yeast. A kap95 null strain harboring a URA3 plasmid expressing Kap95 was transformed with plasmids containing LexA, LexA-importin-β, LexA-I178D-importin-β, or LexA-KAP95. Complementation was assayed on synthetic complete media containing 2% glucose and 5-fluoroorotic acid at 23 °C for 7 days. Expression of the LexA constructs and the absence of Kap95 in cells expressing LexA-importin-β were confirmed by immunoblot.

Fig. 7. Soluble GLFG inhibits nuclear protein import in permeabilized cells. Soluble FxFG (Nsp1 FF18) at 10 μM (B) and GLFG (GST-Nup100 (2-610)) at 25 μM (C) both inhibit importin-β-dependent import of a fluorescein-labeled NLS-BSA substrate (A). Bar is 25 μm.

DISCUSSION

The preferential association of individual transport factors with different FG-Nups has raised the possibility that there are functionally different pathways for movement through NPCs (Ref. 11, reviewed in Ref. 17). Data obtained using the S. cerevisiae importin-β homologue Kap95 suggested that GLFG- and FxFG repeat-containing Nups may be involved in different steps of the transport pathway (22). To address the extent to which these different classes of Nups might contribute to different trafficking pathways, we have explored the way in which each biochemically interacts with importin-β.

The crystal structures of the Ib442-GLFG and Ib442-FxFG complexes (Fig. 2), the dramatically reduced affinity of I178D-importin-β for both FxFG and GLFG repeats (Figs. 3 and 4), and the ability of FxFG repeats to displace GLFG repeats from importin-β (Figs. 3 and 5) are all consistent with both classes of FG-Nups binding to overlapping sites on importin-β. Moreover, both the crystal structures and the reduced affinity for both FxFG and GLFG repeats shown by the I178D mutant indicate that a common binding site is located in the hydrophobic pocket between the A-helices of HEAT repeats 5 and 6. Finally, the
binding data and competition experiments with Kap95 (Figs. 4 and 5) were analogous to those obtained with importin-β.

There are unexpected similarities in the way in which each type of repeat binds importin-β. The structural data indicate that the core hydrophobic residues of both types of repeat make major contributions to the interaction with importin-β. The Phe side chain of the FG dipeptide from solvent. Although in contrast, the hydrophobic residue in the first half of the repeat core (Leu or Phe) appears to function primarily to shield the Phe side chain of the FG dipeptide from solvent. Although our structural data do not exclude the possibility that there may be additional contributions to the binding to importin-β from residues in the hydrophilic linkers between the GLFG and FxFG cores, especially in the regions immediately flanking the cores, these are probably not major determinants of binding. However, by analogy to the results obtained with G-proteins binding to their effectors (see, for example, Ref. 56), interactions with these flanking regions might make contributions to the selectivity of interactions or to modulating the affinity of interactions between carriers and Nups. For example, this could explain why different carrier molecules may have preferences for different portions of the Nup116 GLFG repeat region (41).

The structural data were obtained using only residues 1–442 of importin-β and a GLFG peptide, which inevitably raises the question of whether the binding site observed is an artifact associated with the use of fragments or with crystallization. However, these results were completely validated by the binding and competition data using full-length importin-β or Kap95 and Nup GLFG and FxFG domains that contained a large number of repeats. These data certainly do not exclude the possibility of additional FxFG or GLFG binding sites on importin-β or Kap95. However, the competition binding experiments indicate that any such binding sites would be of lower affinity than the site between the A-helices of HEAT repeats 5 and 6. Previous deletion mutagenesis studies have indicated that a major NPC binding site on importin-β is located between residues 152 and 352 (and thus between the A-helices of HEAT repeats 5 and 6), but at least one additional NPC binding site, probably with lower affinity, may exist between this site and residue 618 of importin-β (21, 31, 32). Based on our new in vitro binding analysis, the previous observations of differential FG-nucleoporin two-hybrid interactions with the Kap95-L63A mutant (22) may not reflect differences in FG nucleoporin binding sites. In the absence of high-resolution structural information for either Kap95 or a complex of Kap95 with FG Nup repeats and further analysis of FG-binding mutants, it is not possible to be certain that the GLFG/FxFG site in Kap95 is directly equivalent to the site characterized in importin-β (Fig. 2 and Ref. 21).

The binding of both FxFG and GLFG repeats to overlapping sites on importin-β raises the question of the extent to which there is a functional difference between FxFG- and GLFG-Nups, at least in the context of importin-β-mediated nuclear protein import. A number of studies have indicated that GLFG and FxFG repeats are not simply interchangeable in vivo and at least some of their functions in trafficking pathways may be distinct. For example, in S. cerevisiae, the FxFG region of Nsp1 cannot substitute for the GLFG region of Nup116 (12). Furthermore, different transport factors appear to preferentially associate with different Nups (Ref. 11, reviewed in Ref. 17), and genetic approaches show that some Nups are required for certain classes of transport, whereas other Nups are dispensable (reviewed by Ref. 17). One way in which GLFG- and FxFG-Nups could share common binding sites on importin-β/Kap95 and yet be functionally different, would be if the different FG-Nup classes were associated with different steps of the nuclear protein import process. In this context, it may be important that different classes of FG-Nups are thought to be distributed differently throughout S. cerevisiae NPCs, with FspFG-Nups located in the cytoplasmic fibrils, FxFG-Nups (except for Nsp1p) located at the nucleoplasmic face, and GLFG-Nups in the central region between them (11, 18). This would be consistent with a model in which the different functions of FxFG- and GLFG-Nups may not be linked so much to their differential interaction with the carrier molecule but rather with their location in the NPC and spatial relationship to the translocation mechanism. Alternatively, the putative lower affinity binding sites may be more functionally significant in vivo than they are in vitro.

Recent work has indicated that, in addition to its central function in nuclear protein import, importin-β also plays an important role in in vitro nuclear envelope assembly with Xenopus egg extracts (57). Based on the observation that the 117D mutant is unable to substitute for wild-type importin-β in this system, it has been proposed (57) that one function of importin-β in nuclear envelope assembly depends on its binding to FxFG nucleoporins. However, our present data demonstrate that the 117D mutant also inhibits the binding of GLFG nucleoporins, and so it is therefore not clear which class of nucleoporin is actually critical for nuclear envelope assembly in the Xenopus system.

In summary, we have shown that GLFG and FxFG nucleoporins both bind to a site between HEAT repeats 5 and 6 of importin-β and that Kap95 similarly binds both classes of Nups at overlapping sites. Although further work will be required to determine whether other importin-β superfAMILY members also bind different types of nucleoporin FG repeats at common sites, the present results indicate that functional differences between different FG-Nups may arise primarily from differences in their spatial organization.

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Importin-βGLFG-nucleoporin Interaction
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