Gradient of Increasing Affinity of Importin β for Nucleoporins along the Pathway of Nuclear Import

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Abstract. Nuclear import and export signals on macromolecules mediate directional, receptor-driven transport through the nuclear pore complex (NPC) by a process that is suggested to involve the sequential binding of transport complexes to different nucleoporins. The directionality of transport appears to be partly determined by the nucleocyttoplasmic compartmentalization of components of the Ran GTPase system. We have analyzed whether the asymmetric localization of discrete nucleoporins can also contribute to transport directionality. To this end, we have used quantitative solid phase binding analysis to determine the affinity of an importin β cargo complex for Nup358, the Nup62 complex, and Nup153, which are in the cytoplasmic, central, and nucleoplasmic regions of the NPC, respectively. These nucleoporins are proposed to provide progressively more distal binding sites for importin β during import. Our results indicate that the importin β transport complex binds to nucleoporins with progressively increasing affinity as the complex moves from Nup358 to the Nup62 complex and to Nup153. Antibody inhibition studies support the possibility that importin β moves from Nup358 to Nup153 via the Nup62 complex during import. These results indicate that nucleoporins themselves, as well as the nucleocytoplasmic compartmentalization of the Ran system, are likely to play an important role in conferring directional-ity to nuclear protein import.

Key words: nuclear import • nuclear pore complex • importin β • Nup62 complex • Nup153

Introduction

Nucleocytoplasmic transport occurs through nuclear pore complexes (NPCs), ~125-mD proteinaceous assemblies that span the nuclear envelope (for reviews see Ohno et al., 1998; Gorlich and Kutay, 1999; Stoffler et al., 1999; Ryan and Wente, 2000). NPCs are composed of ~50–100 different proteins called nucleoporins. The major framework of the NPC consists of eight spokes, which are flanked by nuclear and cytoplasmic rings that surround a central channel structure. Extending outward from the ring–spoke assembly are ~35–50-nm-long cytoplasmic fibrils and ~50–100-nm-long nuclear fibrils that are joined in a basket-like structure. Ions, metabolites, and small proteins (<20–40 kD) move through the NPC by passive diffusion, but most larger molecules are transported by signal- and energy-dependent mechanisms. Most signal-dependent nuclear transport is mediated by nucleoplasmic shuttling receptors of the importin/karyopherin β family (for reviews see Ohno et al., 1998; Gorlich and Kutay, 1999). Nuclear localization signals (NLSs) or nuclear export signals in protein or RNA cargo are recognized directly by transport receptors or indirectly via adaptor proteins that bind to the receptors. After a receptor–cargo complex is formed, it is translocated through the NPC by a multistep process that apparently involves the sequential binding of the transport receptor to nucleoporins in different NPC regions. Cargo is then released from the receptor and the latter is recycled. The small GTPase Ran, which interacts with importin β-type receptors, plays a key role in driving both nuclear import and export (see below).

The classical NLS is characterized by a basic amino acid–rich sequence, which is present in a simple or bipartite motif. This NLS is recognized by the adaptor importin α, which binds to the import receptor importin β through its importin β binding (IBB) domain. Importin β–related receptors have been shown to bind directly to several nucleoporins that contain FG repeat motifs, and these bind-
Materials and Methods

Expression and Purification of Recombinant Proteins

All proteins were expressed in *Escherichia coli* BL21 (strain DE3) as described previously: glutathione S-transferase (GST)-tagged Nup62, Nup8, and Nup54 (Hu et al., 1996), Nup358 fragments (358-1 and 358-4) (Yaseen and Blobel, 1999), 6× his-S-tagged importin β (Chi and Adam, 1997), 6× his-tagged importin α (Hu et al., 1996), 6× his-tagged IBB domain (Weis et al., 1996), nuclear transport factor 2 (Paschal and Gerace, 1995), Ran and RanG69L (Melchior et al., 1995), and RanBP1 (Kehlenbach et al., 1999). An expression clone for the 6× his-tagged COOH-terminal segment of human Nup153 comprising amino acids 609–1475 was constructed by subcloning an XbaI-BglII fragment into the blunt HindIII site of PET28a (Novagen). An expression clone for full length 6× his-tagged human Nup98 was constructed by subcloning a HindIII–XhoI fragment into the same restriction sites of PET28b. Cells transformed with C-153 or Nup98 were grown at 37°C to an OD_{600} of 0.6. Protein was induced with 1 mM IPTG for 3 h and was purified on Talon beads (CLONTECH Laboratories, Inc.).

**Nuclear Import Assays**

The nuclear import assay was carried out in NRK cells supplemented with HeLa cytosol. For the antibody inhibition assay, NRK cells were trypsinized and washed with transport buffer (TB: 20 mM Hapes, pH 7.4, 110 mM KOAc, 2 mM MgOAc, 2 mM DTT, 1 μg/ml of pepstatin, leupeptin, and aprotinin). The cells were then permeabilized with digitonin (Adam et al., 1992) and subsequently incubated with 10 μg/ml RanG69L and 10 μg/ml RanBP1 for 15 min at 30°C to deplete them of endogenous importin β (Kehlenbach, R., personal communication). The cells were then preincubated on ice with 0.5 mg/ml anti-Nup62 Fab fragment or with an equivalent volume of TB, and nuclear import was carried out and quantified by flow cytometry described (Melchior et al., 1998).

To analyze the binding of his–S–importin β to nucleoporins in cells preincubated with the anti-Nup62 Fab fragment, cells were incubated with 73.5 nM his–S–importin β, 330 nM his–importin α, 500 nM Ran, 670 nM nuclear transport factor 2, 25 μg/ml FITC-NLS-BSA and an energy regenerating system at 30°C for 30 min. Next, the cells were washed with TB, and a lysate prepared with NP-40 buffer containing 0.3 M NaCl (Kehlenbach et al., 1999) was subsequently incubated with S-protein–agarose. The bound proteins were analyzed by SDS-PAGE followed by immunoblotting with affinity-purified anti-Nup153 and anti-Nup358 antibodies. To analyze the effect of anti-Nup62 Fab fragment on the interaction between recombinant Nup62 and importin β, GST-Nup62 absorbed to glutathione beads was preincubated with the anti-Nup62 Fab fragment. Beads were then incubated with importin β and analyzed by SDS-PAGE.

**Preparation and Affinity Purification of Antibodies**

For antibody production, Nup62 was expressed as an untagged protein in *E. coli* and purified as described (Paschal and Gerace, 1995), with an additional step involving chromatography on a DEAE column with a 0–0.3 M NaCl gradient. Nup62 and a keyhole lymphocyanin conjugate of amino acids 921–930 of Nup153 were used to immunize rabbits. The resulting antibodies were affinity purified on a resin coupled to Nup62 or to the Nup153 peptide. The Fab fragment of the anti-Nup62 antibodies was prepared using papain-agarose beads (Pierce Chemical Co.).

**Microtiter Plate Binding Assay**

Solid phase binding assays were carried out on microtiter plates (Maxisorp; Nunc) coated with 25 ng of nucleoporin. Assays were conducted as described (Delphin et al., 1997) except the bound his–S–importin β was detected using anti-S tag antibodies (CLONTECH Laboratories, Inc.) and horseradish peroxidase-conjugated secondary antibodies (Pierce Chemical Co.). Colorimetric detection was carried out using 3,3′, 5,5′-tetramethylbenzidine (Calbiochem). Values were corrected for background binding of importin β to GST alone. For 6× his–tagged proteins, wells adsorbed with BSA served as the control. To study the effect of RanGTP on the binding of importin β to nucleoporins, recombinant Ran was loaded with GTP and used for binding assays (Delphin et al., 1997).

**Results and Discussion**

**Characterization of the Binding of Importin β and an Importin β-IBB Complex to Nucleoporins**

To investigate whether the affinity of the importin β transport complex for nucleoporins changes as the complex moves through the NPC, we carried out quantitative solid
phase binding analysis with several FG repeat nucleoporins that are relatively abundant components of the NPC (Snow et al., 1987) and that have been shown previously to interact with importin β in qualitative assays. We analyzed Nup358 (Yaseen and Blobel, 1999), which is in the cytoplasmic fibrils, the Nup62, Nup58, and Nup54 subunits of the Nup62 complex (Hu et al., 1996), which are near the central channel of the NPC, and Nup153 (Shah et al., 1998), which is in the nucleoplasmic fibrils. Based on their localization, these proteins are predicted to be involved in early, intermediate, and late steps of transit through the NPC, respectively. In these binding studies, we analyzed full length Nup62, Nup58, and Nup54. Since it currently is not possible to obtain recombinant full length Nup358 and Nup153, we examined two FG repeat–containing fragments of Nup358 (Nup358-1, amino acids 996–1963; Nup358-4, amino acids 2500–3224) and the COOH-terminal region of Nup153 (Nup153-C, amino acids 609–1475).
that contains the only detectable binding site for importin β (Shah et al., 1998).

The binding experiments were conducted both with importin β alone and with importin β bound to the IBB domain of importin α (Fig. 1 and Table I). The IBB domain behaves as an authentic import cargo for importin β and closely resembles certain NLSs that bind to importin β in an importin α–independent fashion (for review see Gorlich and Kutay, 1999). The binding isotherms for each of the six proteins tested showed saturable binding of both importin β and the importin β–IBB domain complex, as evidenced by linear double reciprocal plots (Fig. 1; data not shown). The apparent affinity of importin β for the nucleoporins tested is similar in the presence and absence of the IBB domain. This argues that the region of importin β involved in nucleoporin binding is not conformationally altered by cargo binding. Interestingly, the affinity of importin β was lowest for each of the Nup358 fragments ($K_d = 210–225$ nM), increased $\sim 2$-fold for each of the Nup62 complex proteins ($K_d = 100–105$ nM), and increased another $\sim 10$-fold for Nup153-C ($K_d = 9$ nM) (Table I). Thus, there is a progressively increasing affinity of importin β for nucleoporins that occur progressively closer to the nucleoplasmic periphery of the NPC.

We also analyzed binding of importin β to Nup98, an FG repeat nucleoporin that does not appear to be required for importin β–mediated import, since import still occurs in nuclei assembled from Xenopus egg extracts depleted of Nup98 (Powers et al., 1995). No specific binding was seen by the microtiter plate binding assay with the concentration range of importin β analyzed for the other nucleoporins (data not shown), as the low level association of importin β with recombinant Nup98 that we observed was nonsaturable and was not inhibited by RanGTP (see below). It should be noted that in a previous study, we did not detect a difference in the affinity of importin β for Nup358 purified from rat liver compared with recombinant Nup62 (Delphin et al., 1997). This difference from our current results may be explained by our finding that
The binding of importin β to Nup358 can occur by two different mechanisms (Delphin et al., 1997; Yaseen and Blobel, 1999). One involves the binding of importin β by itself to FG repeat regions of Nup358, and is blocked in the RanGTP–importin β complex. The second mechanism, which is higher affinity, involves the binding of the importin β–RanGTP complex to the Ran binding domains (RBDs) of Nup358 via RanGTP. We measured the binding of importin β to the two fragments of Nup358 described above (Fig. 2, E and F). We found that a 2:1 ratio of RanGTP/importin β enhanced the binding of the importin β to the Nup358 fragments, as expected by the presence of one RBD in each fragment. By contrast, a 10:1 ratio diminished the binding, apparently reflecting the competition of free RanGTP with the RanGTP–importin β complex for the RBDs. These results agree with previous findings made with intact Nup358 (Delphin et al., 1997).
Nup153 that coprecipitated with importin β in the Fab fragment-treated cells as compared with the control (Fig. 3 B). Immunofluorescence microscopy demonstrated that the binding of anti-Nup62 antibodies did not dissociate Nup153 from the NPC (data not shown).

Because the solubilization conditions we used for immunoprecipitation released only a small fraction of the Nup62 complex from the permeabilized cells, we were unable to measure the association of importin β with the Nup62 complex in this experiment. However, using an alternative approach, we found that the anti-Nup62 Fab fragment strongly diminished the amount of importin β bound to column-immobilized recombinant GST-Nup62 in vitro compared with the untreated control (Fig. 3 C). This suggests that the antibody may inhibit import in permeabilized cells by blocking a binding site on Nup62 for importin β, although it cannot be excluded that the antibody acts by sterically inhibiting the binding to another nearby subunit protein of the Nup62 complex. Considered together, our data argue that the Nup62 complex is directly involved in nuclear import, and that the complex is an intermediate NPC binding site for importin β as it traverses the NPC between binding sites at Nup358 and Nup153.

**Implications for the Mechanism of Nuclear Protein Import**

Several models for the movement of transport complexes through the NPC have been discussed (Rexach and Blobel, 1995; Nachury and Weis, 1999). We believe that the simplest model for importin β-mediated nuclear import that is consistent with the observations presented in this study is an “affinity gradient” mechanism (Fig. 4). Our antibody inhibition and biochemical analyses support the possibility that movement of importin β through the NPC involves its transfer from Nup358 to Nup153 via the Nup62 complex. Although other unidentified nucleoporin intermediates may also be involved, it is possible that Nup358 and Nup153, which are components of the flexible cytoplasmic/fibrils, might be able to directly interact with the Nup62 complex. Based on the progressive increase in the affinity of importin β for Nup358, Nup62 complex proteins, and Nup153, we suggest that the movement of the importin β cargo complex through the NPC has a strong cytoplasmic-to-nuclear directional bias due in part to increasing affinity of the transport complex for the nucleoporin binding sites that it sequentially encounters. In the simplest situation, transfer between nucleoporin pairs could occur in either a forward or backward direction at each step, but forward movement would be favored by an increase in the affinity of transport complexes for more distal nucleoporins. Release from the terminal nucleoporin binding site could be mediated by RanGTP (Gorlich et al., 1996). It is striking that two different FG repeat regions of Nup358 bound to importin β with nearly identical affinity, as did three different subunits of the Nup62 complex. This suggests that the affinity of import complexes for a specific region of the NPC may be an important parameter in specifying directionality (see below).

Precisely how the transport complex is transferred between two nucleoporins is unclear. In one scenario, this could be a concerted reaction whereby an importin β cargo complex bound to one nucleoporin is induced to release from the first binding site upon interacting with a second nucleoporin. Consistent with this possibility, importin β may have at least two distinct binding sites for nucleoporins (Kutay et al., 1997). We attempted to investigate this model by monitoring the ability of Nup153 to release importin β from Nup62, and by the ability of Nup62 to release importin β from the Nup358 fragments. Unfortunately these experiments were not informative, since under our experimental conditions Nup153, Nup62, Nup58, and the Nup358 fragments interact with each other (our unpublished results).

The on-rate for association of importin β with nucleoporins may be in the range of $10^7$–$10^8$ M$^{-1}$ s$^{-1}$ (Berg and Von Hippel, 1985; Chaillan-Huntington et al., 2000), and so the measured dissociation constants for importin β binding to Nup358, to the Nup62 complex, and to Nup153 would imply off-rates on the order of 2–20/s, 1–10/s, and 0.1–1/s, respectively. Since the rate of nuclear transport is thought to be in the range of 10–100 events/s (for review see Gorlich and Kutay, 1999), it would seem that these interactions, especially the binding to Nup153, would predict an interaction persistence time that is too long to support import by simple on/off binding reactions. However, much more rapid transfer between nucleoporin pairs could occur at each step if a concerted transfer were involved (see above). Moreover, additional factors, such as RanGTP (Rexach and Blobel, 1995; Gorlich et al., 1996), could promote the transfer/release reactions. An analogous mechanism involving a gradient of increasing nucleoporin bind-

![Figure 4. Model for the directional movement of an importin β cargo complex through the NPC. See text for details.](image-url)
importing affinity may function in nuclear export, to promote directional movement of export complexes from the nucleus to the cytoplasmic surface of the NPC. For example, chromosome maintenance region 1-containing nuclear export complexes have a substantially higher affinity for the cytoplasmic fibril protein Nup214/CAN than for more proximal nucleoporin binding sites in the export pathway including the Nup62 complex and Nup153 (Kehlenbach et al., 1999; Kehlenbach, R., and L. Gerace, unpublished). Finally, a similar mechanism may be involved in the recycling of importin β to the cytoplasm after import, since the RanGTP–importin β complex that is thought to be created in the nucleus by dissociation of the import complex has a much higher affinity for the cytoplasmic periphery of the NPC than for any other NPC region (Delphin et al., 1997).

In conclusion, we propose that the increasing affinity of importin β for nucleoporins that are localized progressively closer to the nucleoplasmic surface of the NPC contributes to the directional movement of import complexes through the NPC. This mechanism would clearly enhance the efficiency of directional nuclear transport that is promoted by compartmentalization of different components of the Ran system.

We thank Dr. S. Lyman, Dr. G. Cingolani, and P.D. Frost for comments on the manuscript, and Drs. N. Yaseen, S.A. Adam, B. Burke, J. Borrow, and K. Weis for providing expression vectors for Nup358, his-S-tagged importin β, Nup153, Nup98, and the IBB domain, respectively.

This work was supported by a fellowship from the Human Frontiers Science Program to I. Ben-Efraim (LT-105/97) and by a grant from the National Institutes of Health to L. Gerace (GM41955).

Submitted: 30 August 2000
Revised: 8 November 2000
Accepted: 2 December 2000

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