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Importin β contains a COOH-terminal nucleoporin binding region important for nuclear transport

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Proteins containing a classical NLS are transported into the nucleus by the import receptor importin β, which binds to cargoes via the adaptor importin α. The import complex is translocated through the nuclear pore complex by interactions of importin β with a series of nucleoporins. Previous studies have defined a nucleoporin binding region in the NH$_2$-terminal half of importin β. Here we report the identification of a second nucleoporin binding region in its COOH-terminal half. Although the affinity of the COOH-terminal region for nucleoporins is dramatically weaker than that of the NH$_2$-terminal region, sets of mutations that perturb the nucleoporin binding of either region reduce the nuclear import activity of importin β to a similar extent (∼50%). An importin β mutant with a combination of mutations in the NH$_2$- and COOH-terminal regions is completely inactive for nuclear import. Thus, importin β possesses two nucleoporin binding sites, both of which are important for its nuclear import function.

Introduction

Nucleocytoplasmic transport occurs through nuclear pore complexes (NPCs; for reviews see Fahrenkrog et al., 2001; Rout and Aitchison, 2001; Vasu and Forbes, 2001), large proteinacious structures that span the nuclear envelope. The NPC has a central framework consisting of eight spokes surrounding a central transport channel, which is flanked by cytoplasmic and nuclear rings. Extending outward from the ring–spoke framework are eight cytoplasmic fibrils, and eight nuclear fibrils that are joined distally to form a nuclear basket. The vertebrate NPC has a mass of up to 125 MD (Reichelt et al., 1990) and consists of ∼30 proteins, which are each present in multiple copies (Cronshaw et al., 2002). About one third of the nucleoporins possess multiple copies of the phenylalanine-glycine (FG) sequence. The FG repeats often are part of larger GLFG or FxFG motifs and are usually clustered in specific nucleoporin domains (for review see Bednenko et al., 2003).

Nucleocytoplasmic transport of proteins and RNAs is commonly mediated by receptors of the importin β/karyopherin β superfamily. The transport receptors recognize NLSs or nuclear export signals (NESs) on cargoes and transfer them through the NPC by interacting with FG repeat nucleoporins (for reviews see Görlich and Kutay, 1999; Kuersten et al., 2001; Macara, 2001; Weis, 2002). Importin β, which is the best-studied nuclear import receptor, interacts with its cargos either directly or via adapters such as importin α. Translocation of importin β into the nucleus appears to involve its interaction with a series of FG repeat nucleoporins, including Nup358, which is located in the cytoplasmic fibrils (Wilken et al., 1995; Yokoyama et al., 1995; Delphin et al., 1997), Nup62 complex proteins (Nup45, Nup54, Nup58, and Nup62), which are present at the central channel (Hu et al., 1996), and Nup153, which is present in the nuclear basket (Sukegawa and Blobel, 1993; Shah et al., 1998).

The small GTPase Ran plays a key role in the directionality of karyopherin-mediated nuclear transport (for reviews see Görlich and Kutay, 1999; Kuersten et al., 2001; Macara, 2001; Weis, 2002). Due to the nucleocytoplasmic compartmentalization of Ran regulators, RanGTP is concentrated in the nucleus and RanGDP in the cytoplasm. In the case of importin α/β–mediated import, RanGTP in the nucleus dissociates importin α from importin β, which promotes cargo unloading. RanGTP may also play an important role in dissociating importin β from nucleoporins during receptor movement through the central channel, as well as in releasing the receptor from Nup153 at the end of translocation (Rexach and Blobel, 1995; Görlich et al., 1996; Lyman et al., 2002). Importin β is recycled to the cytoplasm in

Abbreviations used in this paper: IBB, importin β binding; NPC, nuclear pore complex; PTHrP, parathyroid hormone–related protein.

complex with RanGTP, where import-competent importin β is regenerated upon RanGTP hydrolysis promoted by RanGAP.

Importin β contains 19 tandem HEAT repeats arranged in a superhelical spiral (Cingolani et al., 1999; Fig. 1 A). Each HEAT repeat, which usually has a length of ∼40–60 amino acid residues, consists of A and B helices connected by a short turn. The A helices form the outer surface of importin β, and the B helices create the inner surface. The inner surface of importin β binds to the ∼45-residue importin β binding (IBB) domain of importin α (via HEAT repeats 7–19; Cingolani et al., 1999), the NLS of parathyroid hormone–related protein (PTHrP) (via HEAT repeats 2–11; Cingolani et al., 2002), and RanGTP (via HEAT repeats 1–8; Vetter et al., 1999). The outer surface of importin β contacts FG repeat motifs of nucleoporins (Bayliss et al., 2000, 2002).

The major NPC binding domain in importin β was mapped by deletion mutagenesis to its NH2-terminal region between HEAT repeats 4–9 (Chi and Adam, 1997; Kose et al., 1997; Kutay et al., 1997). In addition, the importin β fragment containing HEAT repeats 9–19 has been shown to bind to the nuclear envelopes of digitonin-permeabilized HeLa cells (Kutay et al., 1997), raising the possibility of additional nucleoporin binding sites in the COOH-terminal part of importin β. The crystal structure of a complex between an NH2-terminal fragment of human importin β (HEAT repeats 1–10) and an FxFG-rich nucleoporin fragment has revealed two FxFG binding sites in the NH2-terminal region of importin β, one formed by HEAT repeats 5 and 6 and another formed by HEAT repeats 6 and 7 (Bayliss et al., 2000). Each of these binding elements forms a pocket that associates with the two phenylalanine residues of the FxFG motif by hydrophobic interactions.

In this study, we present an in-depth biochemical analysis of the interaction between importin β and nucleoporins. We prepared a number of point mutations in the NH2-terminal region of importin β previously implicated in nucleoporin binding. Although the mutations diminished nucleoporin binding in a manner consistent with the predictions of the crystal structure (Bayliss et al., 2000), they had a much weaker effect on the level of nuclear import than reported previously (Bayliss et al., 2000). This led us to search for an additional nucleoporin binding site(s) in importin β. Using intramolecular structural alignment of importin β segments and site-directed mutagenesis, we identified a novel nucleoporin binding region in the COOH-terminal half of importin β. Although the COOH-terminal nucleoporin binding region has a dramatically lower affinity for nucleoporins than the NH2-terminal region, it appears to contribute equally to the nuclear import function of importin β. We discuss the possibility that the presence of multiple nucleoporin binding regions is a general feature of nuclear transport receptors.

Results

Effects of single point mutations in the NH2-terminal region of importin β on binding to nucleoporins

Previous studies reported that isoleucine 178 in HEAT repeat 5 is critical for importin β function, because substitution of isoleucine 178 with alanine, aspartate, or phenylalanine was observed to almost completely abolish the nucleoporin binding and nuclear import activity of importin β (Bayliss et al., 2000, 2002). Surprisingly, when we prepared the I178A and I178D importin β mutants, we discovered that these mutations had little or no effect on nuclear import (see below). Therefore, we performed a systematic mutational analysis of the nucleoporin binding region in HEAT repeats 5–7 to evaluate the importance of other importin β residues in this region. Our goal was to identify combinations of single point mutations that significantly reduce the ability of importin β to interact with nucleoporins and to support nuclear import, without diminishing the interaction with cargo or with the transport regulator Ran.

Figure 1. **Model of the importin β–IBB domain complex.** (A) Rod representation of the structure of importin β complexed with the IBB domain of importin α, according to Cingolani et al. (1999). The A and B helices of the HEAT repeats are shown in red and yellow, respectively, with connecting loops and helices in gray. The IBB domain of importin α is in green. (B) An enlarged view highlighting the A helices of HEAT repeats 5–7, which were shown to be involved in binding FxFG motifs by crystallography (Bayliss et al., 2000). The enlarged image was rotated by 90° in a Y direction with respect to A. The side chains of the residues analyzed by the mutagenesis as described in Table I are highlighted in blue.
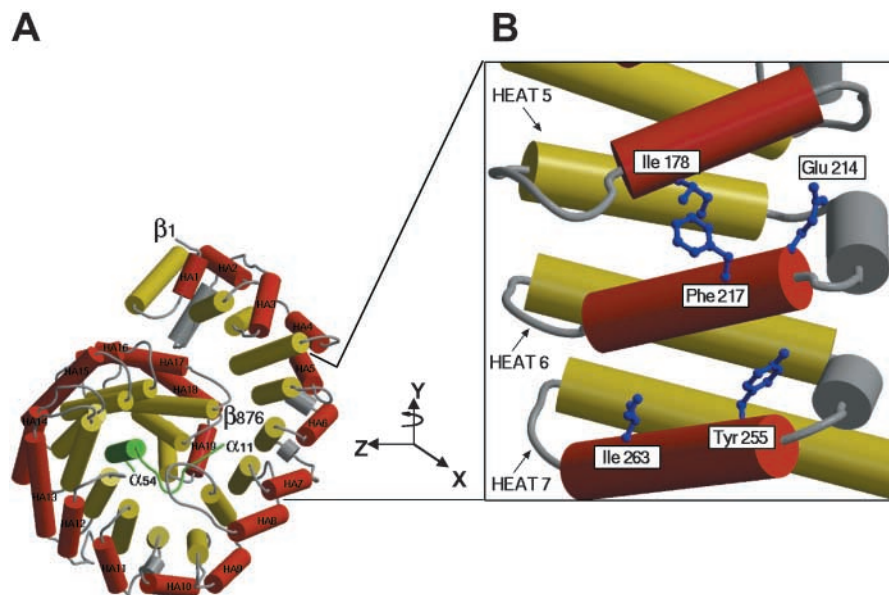
Table I. **Effect of mutations in HEAT repeats 5–7 on the affinity of importin β for nucleoporins**

Importin β	HEAT repeat region[a]	K_d, Nup153 (895–1475)	K_d, Nup62	K_d, Nup358 (996–1963)	K_d, RanGTP	K_d, importin α
		nM	*nM*	*nM*	*nM*	*nM*
WT	NA	1.1 ± 0.1	77.3 ± 11.6	190.2 ± 18.7	3.3 ± 0.3	0.9 ± 0.1
I178A	5A	1.1 ± 0.1	89 ± 9	245 ± 34	3.6 ± 0.3	1.1 ± 0.1
I178D	5A	**40.1 ± 9.1**	NS	NS	4.7 ± 0.6	1.2 ± 0.1
E214A	6A	1.8 ± 0.1	**770.4 ± 123.2**	NS	3.1 ± 0.3	1.3 ± 0.1
F217A	6A	2.1 ± 0.2	227.4 ± 33.3	NS	4.1 ± 0.5	0.7 ± 0.2
Y255A	7A	3.5 ± 0.3	NS	NS	3.0 ± 0.5	0.9 ± 0.2
I263R	7A	2.5 ± 0.3	NS	NS	3.9 ± 0.2	0.9 ± 0.1
I178A/E214A	5A, 6A	**27.0 ± 4.1**	NS	NS	5.1 ± 0.6	0.8 ± 0.1
F217A/Y255A	6A, 7A	**32.4 ± 5.3**	NS	NS	4.4 ± 0.6	0.8 ± 0.1
I178A/F217A/Y255A/I263R (m-N)	5A, 6A, 7A	**99.0 ± 15.2**	NS	NS	3.9 ± 0.4	1.2 ± 0.1
I178D/Y255A	5A, 7A	**68.3 ± 9.0**	NS	NS	**44.1 ± 6.2**	1.1 ± 0.2

Apparent K_d values that differ from the wild-type values by more than fivefold are shown in bold. NA, not applicable; NS, nonsaturated binding at importin β concentrations up to 800 nM (high nonspecific binding to a GST control protein was observed at importin β concentrations of >800 nM, precluding the accurate measurement of a K_d in these cases).
[a]HEAT repeat 5, residues 170–211; HEAT repeat 6, residues 212–252; HEAT repeat 7, residues 253–313.

According to the crystal structure of a complex between an NH₂-terminal fragment of human importin β (HEAT repeats 1–10, comprising amino acid residues 1–442) and an FxFG-rich fragment of the yeast nucleoporin Nsp1p (residues 497–608), importin β contains two binding sites for the FxFG motif within HEAT repeats 5–7 (Bayliss et al., 2000). One site is formed by residues in HEAT repeats 5 and 6, and a second site by residues in HEAT repeats 6 and 7 (Fig. 1). The first phenylalanine residue of the FxFG motif forms a stacking interaction with either F217 on HEAT repeat 6 (site 1) or Y255 on HEAT repeat 7 (site 2). The second phenylalanine residue of the FxFG motif interacts with a hydrophobic pocket generated by the residues of either HEAT repeats 5 and 6 (site 1; L174, T175, I178, E214, F217, and I218) or HEAT repeats 6 and 7 (site 2; E224, A259, and C223).

Based on predictions of the crystal structure, we made substitutions in residues I178, E214, F217, Y255, and I263 (Fig. 1; Table I). Alanine substitutions of residues F217 and Y255 are expected to abolish the stacking interactions with the first phenylalanine of the FxFG repeat. Furthermore, the mutations I178D, I178A, and E214A are expected to change the structure of the hydrophobic pocket between HEAT repeats 5 and 6 and, in the case of E214A, to remove a hydrogen bond with the mainchain nitrogen of glycine in the FxFG motif (Bayliss et al., 2000). Although I263 itself was not predicted to form contacts with the FxFG repeat (Bayliss et al., 2000), we expected that its substitution with arginine would perturb the apolar pocket between HEAT repeats 6 and 7 located in the vicinity of I263.

We analyzed recombinant importin β mutants for their interaction with several mammalian nucleoporins implicated in nuclear import using an ELISA-based microtiter plate binding assay to measure the apparent dissociation constants (K_ds) (Delphin et al., 1997; Ben-Efraim and Gerace, 2001). We examined a fragment of Nup358 (residues 996–1963) that has 10 FG repeats, six occurring within an FxFG motif; full-length Nup62 that has six FG repeats, five within an FxFG; and a COOH-terminal fragment of Nup153 (resi-

dues 895–1475) that has 25 FG repeats, 16 within an FxFG (for review see Bednenko et al., 2003).

We found that importin β single point mutants containing the substitutions I178D, E214A, F217A, Y255A, and I263R all had a decreased affinity for the Nup153 fragment, but retained wild-type affinity for RanGTP and for importin α (Table I). Weakened nucleoporin binding was most evident for the I178D mutant, which had an ∼40-fold decrease in affinity for Nup153 (895–1475). In contrast, the E214A, F217A, Y255A, and I263R mutants showed a relatively small decrease in affinity for the Nup153 fragment (K_d ≈ 1.8–3.5 nM) (Table I; Fig. 2). All of these mutants had a decreased affinity for Nup62 and Nup358 (996–1963) as well. In several cases, binding of the mutants to Nup62 and Nup358 (996–1963) did not reach saturation in our assay (Table I; Fig. 2), implying a K_d of >>1 μM for the interaction (see Table I legend).

It was reported previously that the I178D and I178A mutations substantially decreased the binding of importin β to nucleoporins (Bayliss et al., 2000, 2002). Although we observed significantly weakened binding with I178D, we found that the I178A mutation had no effect. This is consistent with the theoretical prediction that substitution of a bulky hydrophobic residue with an alanine residue would not significantly change the structure of the hydrophobic pocket. The discrepancy between our results and the previously published study (Bayliss et al., 2000) may be due to differences in the importin β expression and purification conditions. Nonetheless, we observed that combining the I178A mutation with the E214A mutation resulted in substantially weaker nucleoporin binding than the E214A mutation alone (see below; Table I).

Effects of combinations of single point mutations on importin β binding to nucleoporins

In an attempt to dramatically weaken or abolish the high-affinity interaction of importin β with Nup153 (895–1475), we examined a number of constructs that had combinations of single point mutations within the NH₂-terminal nucle-
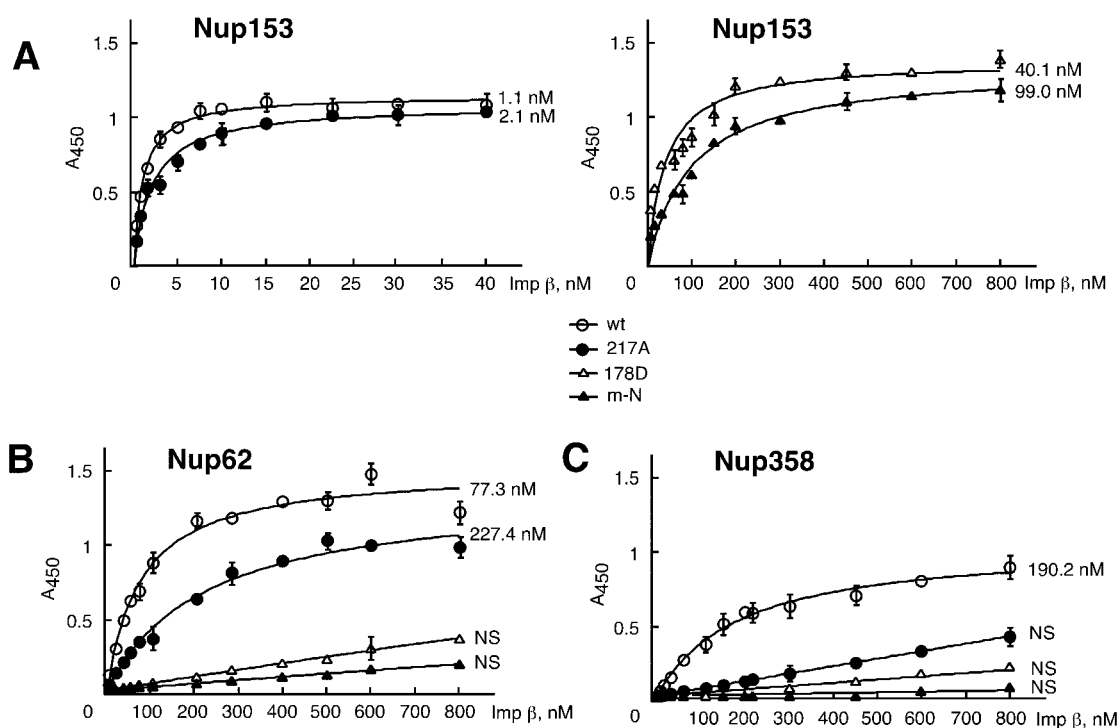
Figure 2. **Characterization of the binding of importin β to nucleoporins.** Shown are binding isotherms depicting the interaction of selected importin β mutants with Nup153 (895–1475) (A), with full-length Nup62 (B), or with Nup358 (996–1963) (C). Error bars represent the standard deviation of duplicate measurements. The calculated apparent K_d values are indicated next to the binding isotherms. NS, nonsaturated binding at importin β concentrations up to 800 nM.

oporin binding region. We found that combining the most potent single point mutation (I178D) with other mutations not only inhibited the interaction with nucleoporins, but also resulted in considerable (>10-fold) inhibition of importin β binding to RanGTP (Table I; unpublished data). For example, importin β containing a combination of I178D and Y255A had an ~13-fold lower affinity for RanGTP, but showed no change in affinity for importin α (Table I). This effect suggests that certain combinations of mutations in the A helices of HEAT repeats 5–7 alter the position of and/or spacing between the residues in the B helices that are important for RanGTP binding, providing evidence for cross-talk between the RanGTP and nucleoporin binding regions (Bayliss et al., 2000).

As our goal was to identify importin β mutants that have significantly reduced affinity for Nup153 yet retain wild-type affinity for RanGTP, we examined combinations of the other single point mutations we had tested. Combining two alanine substitutions in importin β synergistically weakened the binding to Nup153 (895–1475) in the two cases we examined. In one construct (I178A/E214A), the mutated residues belonged to the same FxFG binding pocket, and, in a second construct (F217A/Y255A), they belonged to the two different pockets (Table I). We found that an importin β construct containing a combination of the I178A, F217A, Y255A, and I263R mutations (termed the "m-N" mutant) had an ~100-fold reduced affinity for Nup153, as compared with wild-type importin β. At the same time, the m-N mutant exhibited wild-type affinity for RanGTP and importin α (Table I), and its circular dichroism spectrum was similar to that of wild-type importin β (unpublished data).

Considered together, these data indicate that the mutations in m-N did not significantly alter importin β structure. Introducing additional mutations (e.g., E214A) into the nucleoporin binding region of the m-N construct resulted in inhibition of its binding to RanGTP (unpublished data), and these mutants were not examined further.

Mutations in the NH₂-terminal nucleoporin binding region of importin β: effects on nuclear import and translocation through the NPC

To test the functional effects of mutations in the NH₂-terminal nucleoporin binding domain of importin β, we examined the mutants in a permeabilized cell nuclear import assay using the fluorescently labeled importin α–dependent import cargo BSA-NLS (Adam et al., 1992). At an optimal concentration of wild-type importin β, we observed an approximately fivefold accumulation of fluorescent cargo in nuclei under standard assay conditions, as compared with control reactions in which ATP was depleted (Fig. 3 A, compare white and black columns). Examination of cells by confocal microscopy revealed that almost all of the cell fluorescence was in the nucleus (see Fig. S1, available at http://www.jcb.org/cgi/content/full/jcb.200303085/DC1), validating the use of flow cytometry for quantification. No significant stimulation of import was observed in the absence of recombinant importin β (Fig. 3 A). A time course of import revealed linear nuclear accumulation of cargo over the 30-min time frame of our experiment with wild-type importin β and with all the importin β mutants (unpublished data). This indicates that the observed variations in the level
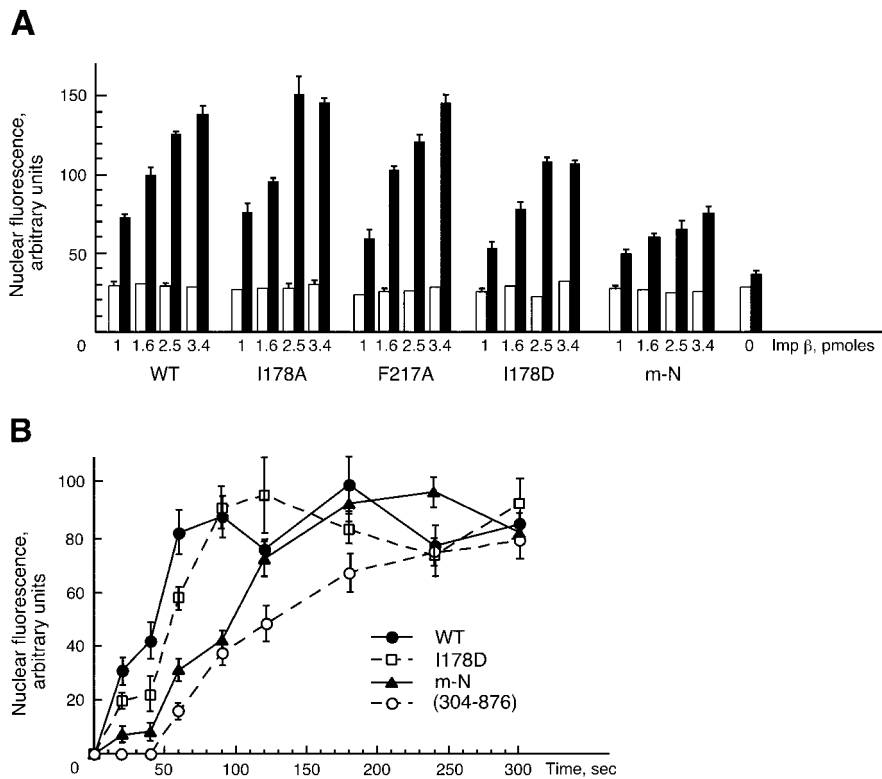
A

B

Figure 3. **Effect of mutations in HEAT repeats 5–7 on nuclear import activity of importin β.** (A) Nuclear import assays with wild-type importin β and with importin β mutants. 40-μl samples were incubated for 30 min at 30°C, and the level of nuclear import of FITC-labeled BSA-NLS was measured by flow cytometry. White bars depict control reactions performed in the presence of hexokinase and glucose to deplete ATP. Black bars show the levels of fluorescence in the presence of ATP. Error bars represent the standard deviation of duplicate measurements. (B) Kinetics of nuclear accumulation of importin β. 3.4 pmol of importin β or importin β (304–876) fragment was used in each 20-μl assay. The level of intranuclear importin β was quantified by immunofluorescence and confocal microscopy in samples fixed at the various time points. Error bars represent the standard deviation of two independent experiments. The level of intranuclear wild-type importin β for the 3-min time point was set at 100%, and all other values were normalized to the 100% level.

of nuclear import with the various importin β constructs are due to import rate differences.

Substitution of wild-type importin β with the I178A mutant did not lower the level of nuclear import, consistent with the wild-type level of nucleoporin binding seen with this mutant (Fig. 3 A). Import with the F217A mutant was decreased by 10–30% at the lower concentrations of importin β tested and was not affected at the higher importin β concentrations examined (Fig. 3 A). The same was true for the E214A, Y255A, and I263R mutants (unpublished data). Import of BSA-NLS with the I178D mutant was reduced by 20–40%, depending on the concentration of the mutant in the assay, whereas import with m-N mutant was reduced by 35–50%. When we performed our assay using the experimental conditions of Bayliss et al. (2000), the nuclear import level was reduced by only 20% with I178D, and no loss of activity was observed with I178A (see Fig. S2, available at http://www.jcb.org/cgi/content/full/jcb.200303085/DC1). This contrasts with the results obtained by Bayliss et al. (2000), who observed a 76–84% loss of import with these two mutants. We think that this discrepancy is due to the different methods of preparation of the recombinant transport factors.

It has been shown that in the absence of exogenous importin α and NLS cargo, importin β can move between the nucleus and the cytoplasm by a process that requires neither Ran nor GTP hydrolysis (Kose et al., 1997, 1999). Similar to this previous work, we found that importin β rapidly accumulated in the nucleus when added to permeabilized cells in the absence of other factors (Fig. 3 B). It quickly reached a plateau concentration, at which point its rate of nuclear entry presumably equals its rate of nuclear exit (Fig. 3 B). To further investigate whether the decrease in cargo import activity of the I178D and m-N importin β mutants is due to a defect

in their ability to be translocated through the NPC, we measured the time course of their nuclear accumulation after addition to permeabilized cells. The same final concentration of intranuclear importin β was achieved with the I178D and m-N mutants, as compared with wild-type importin β (Fig. 3 B). Whereas wild-type importin β reached a plateau within ∼1 min, the I178D mutant took slightly longer to reach this level, and the m-N mutant reached a plateau only after ∼3 min (Fig. 3 B). This suggests that the mutant importin β constructs have a decreased rate of translocation through the NPC. Moreover, their apparent rate of translocation is correlated with their relative nuclear import activity, with the m-N mutant being the most highly impaired.

Nucleoporin binding and NPC translocation of a COOH-terminal fragment of importin β

The nucleoporin binding of the m-N protein that remained in this quadruple mutant (K_d of ∼100 nM for Nup153 [895–1475]) and its significant, albeit reduced, nuclear import activity (∼50% of wild type) could have been due to residual activity in the mutated NH_2-terminal region. It was also possible that an additional nucleoporin binding site(s) in the COOH-terminal region of importin β contributed to nucleoporin binding and import. To systematically investigate these possibilities, we first examined a recombinant NH_2-terminal fragment prepared from the m-N importin β mutant (HEAT repeats 1–10, residues 1–396). We observed that this construct had a K_d of 160 nM for Nup153 (895–1475) (Table II), clearly showing that the m-N mutations did not inactivate all nucleoporin binding in the NH_2-terminal portion of importin β.

We subsequently expressed a COOH-terminal fragment of wild-type importin β that spanned HEAT repeats 8–19

Table II. **Binding of importin β fragments and mutants to Nup153 (895–1475) and to importin α**

Importin β	K_d, Nup153 (895–1475)	K_d, importin α
	nM	*nM*
WT	1.0 ± 0.1	0.9 ± 0.1
1–396	1.2 ± 0.1	ND
m-N (1–396)	160.1 ± 25.3	ND
304–876	152.2 ± 17.5	2.5 ± 0.2
m-C (304–876)	NS	4.3 ± 0.5
m-N	91.3 ± 11.8	1.1 ± 0.1
L612D/F688A (m-C)	1.2 ± 0.1	2.2 ± 0.3
m-N/m-C	83.2 ± 9.1	2.1 ± 0.2

The binding reactions with importin α were performed in PBS containing 30 mg/ml BSA (see Materials and methods). NS, nonsaturated binding at importin β concentrations up to 800 nM.

(residues 304–876). We found that this COOH-terminal importin β fragment bound saturably to Nup153 (895–1475) with a K_d of 152 nM (Table II). However, we were not able to detect an interaction of the COOH-terminal fragment with Nup358 (996–1963) and Nup62, although it is possible that it interacts with these nucleoporins with an affinity that is below the limit of sensitivity of our assay. The affinity of the fragment for importin α was weaker by approximately threefold (Table II), but this can be ascribed to the loss of several amino residues in HEAT repeat 7 that interact with the importin α IBB domain (Cingolani et al., 1999). The fact that the COOH-terminal importin β fragment is still capable of high-affinity binding to importin α suggests that it preserves most of its native structure when expressed as a fragment.

Interestingly, when the COOH-terminal fragment of importin β (304–876) was added to permeabilized cells, it accumulated in the nucleus and reached the same concentration as full-length importin β, although its rate of entry was significantly reduced as compared with the wild-type protein (Fig. 3 B). In addition, importin β (304–876) supported in vitro nuclear import of BSA-NLS in the presence of importin α (see Fig. S3, available at http://www.jcb.org/cgi/content/full/jcb.200303085/DC1). Nuclear import mediated by importin β (304–876) was inhibited by WGA (Fig. S3), suggesting that it was a nucleoporin-mediated process. However, the level of import obtained with the importin β (304–876) fragment was only 30–35% as compared with wild-type importin β, which may be due to the absence of the NH₂-terminal nucleoporin binding domain, the RanGTP binding domain, or both.

Involvement of the COOH-terminal nucleoporin binding region in importin β–mediated nuclear import

To further analyze the nucleoporin binding site contained within the COOH-terminal fragment of importin β, we attempted to identify the COOH-terminal amino acid residues involved in nucleoporin binding. Initially, we performed an intramolecular structural alignment of the NH₂-terminal (HEAT repeats 1–10) and the COOH-terminal (HEAT repeats 11–19) fragments of importin β (Fig. 4, A and B). The two polypeptide chains were aligned using the combinatorial extension (CE) method, which determines an optimal alignment between fragment pairs (Shindyalov and Bourne, 1998). Interestingly, we found that the importin β regions containing residues 1–445 and 446–876 were structurally similar and could be superimposed with a root mean square deviation for the α carbon chains of ∼3.4 Å (Fig. 4 B). The central HEAT repeats (4–7 and 13–16) presented the highest structural homology (root mean square deviation for the α carbon chains of ∼2.1 Å), whereas the HEAT repeats located to the periphery of these core regions showed the highest divergence (Fig. 4 B).

Using this structural alignment, we identified three amino acid residues in HEAT repeats 14 and 16 (L612, F688, and
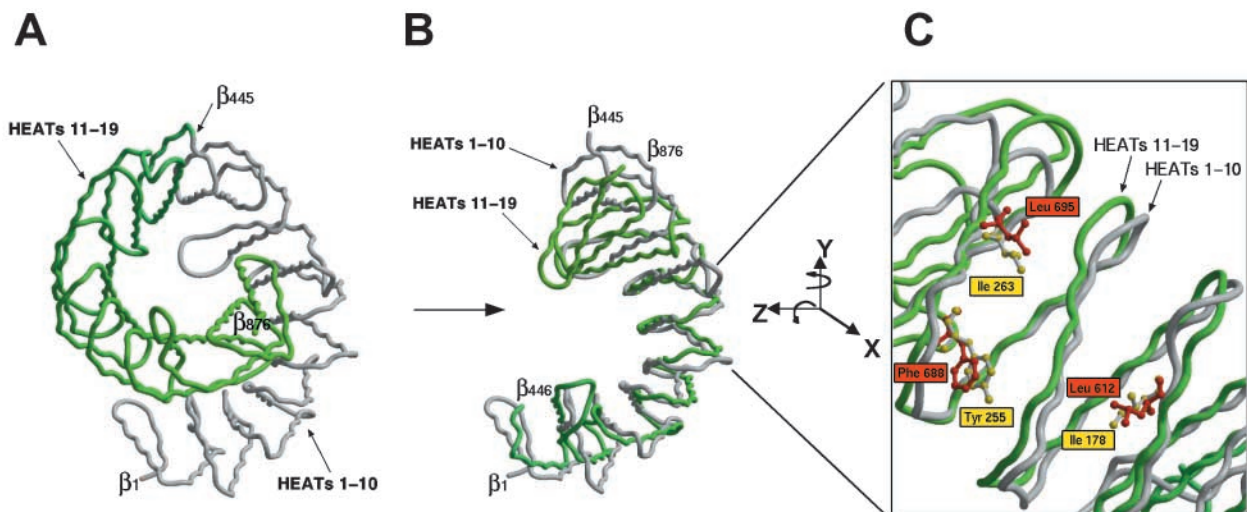

Figure 4. **Structural alignment of the NH₂- and COOH-terminal segments of importin β.** (A) Worm representation of importin β. Residues 1–445 are highlighted in gray and residues 446–876 are in green. (B) Alignment of the NH₂- and COOH-terminal segments of importin β. Residues 1–445 (HEAT repeats 1–10) are in gray and residues 446–876 (HEAT repeats 11–19) are in green. (C) The side chains of importin β residues I178, Y255, and I263 (yellow) closely align with those of L612, F688, and L695 (red), respectively. The enlarged image in C was rotated 90° in a Y direction and 30° in a Z direction with respect to B.
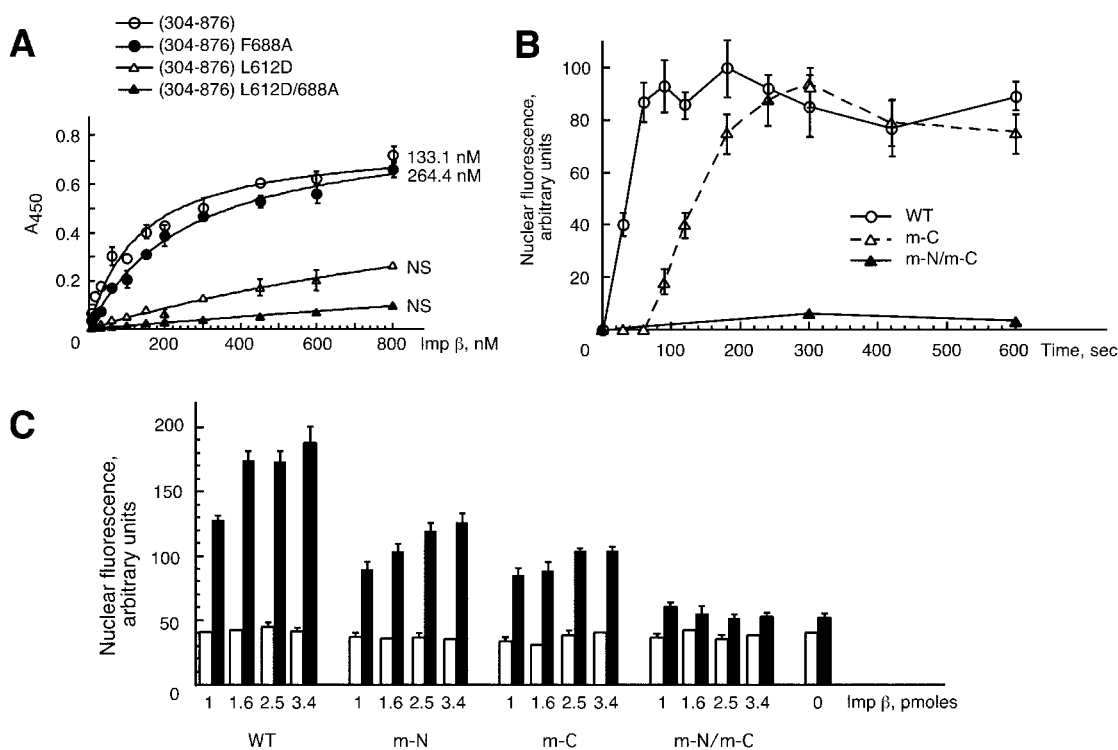
Figure 5. **Effects of mutations in the COOH-terminal segment of importin β on nucleoporin binding and nuclear import.** (A) Binding isotherms showing the interaction of the importin β fragment comprising residues 304–876 (with or without the mutations L612D and/or F688A) to Nup153 (895–1475). Error bars represent the standard deviation of duplicate measurements. The numbers next to the binding curves indicate apparent dissociation constants. NS, nonsaturated binding at importin β fragment concentrations of up to 800 nM. (B) Kinetics of nuclear accumulation of wild-type (WT), m-C, and m-N/m-C full-length importin β proteins. 3.4 pmol of each importin β construct was used in each 20-μl assay. The nuclear fluorescence was measured by immunofluorescence and confocal microscopy after 20–600 s incubation at room temperature, as indicated. Error bars represent the standard deviation of two independent experiments. The level of intranuclear wild-type importin β for the 3-min time point was set at 100%, and all other values were normalized as a fraction of the 100% level. (C) Nuclear import assays with the wild-type (WT), m-N, m-C, and m-N/m-C full-length importin β proteins. 40-μl reactions were incubated for 30 min at 30°C, and the level of nuclear import was measured by flow cytometry. White bars designate control reactions performed in the presence of hexokinase and glucose to deplete ATP. Black bars show the level of fluorescence in the presence of ATP. Error bars represent the standard deviation of duplicate measurements.

L695) that matched residues in the NH2-terminal nucleoporin binding site in HEAT repeats 5 and 7 (I178, Y255, and I263, respectively; Fig. 4 C). To test whether L612, F688, and L695 are important for the interaction of the COOH-terminal fragment of importin β with nucleoporins, we performed a mutational analysis of the three residues. The mutation L612D (analogous to I178D) strongly decreased the affinity of the importin β fragment for Nup153 (895–1475), such that the K_d was not measurable, whereas F688A (analogous to Y255A) decreased the affinity by approximately twofold (Fig. 5 A). The mutation L695R (analogous to I263R) had no detectable effect on the binding of the importin β fragment to Nup153 (unpublished data). A mutant importin β fragment containing a combination of L612D and F688A showed an even lower level of nonsaturated binding for Nup153 (895–1475) than did L612D alone (Fig. 5 A). The L612D and F688A mutations did not significantly affect importin β folding, as only an approximately twofold decrease in the affinity of the mutant protein fragments for importin α was observed (Table II).

We then analyzed the effect of L612D and F688A in the context of full-length importin β. Importin β containing the mutations L612D and F688A (m-C mutant) displayed wild-type affinity for the three nucleoporins tested in this study (Table II; unpublished data). This is expected, as the presence of the high-affinity NH2-terminal nucleoporin binding region in full-length importin β would mask mutational inactivation of the low-affinity COOH-terminal region. We then introduced the L612D/F688A mutations into the m-N mutant to create the m-N/m-C mutant. The affinity of the latter for Nup153 was similar to the affinity of the m-N mutant alone (Table II). This can be explained by the fact that the NH2-terminal nucleoporin binding region in m-N is capable of binding to Nup153 independently of the COOH-terminal nucleoporin binding region (Table II). Both the m-C and the m-N/m-C mutants retained a high affinity for importin α (only approximately twofold weaker than wild type; Table II) and did not exhibit any major alterations in secondary structure, as determined by circular dichroism spectroscopy (unpublished data).

To test the effect of the mutations in the COOH-terminal nucleoporin binding region on importin β function, we first examined the ability of the m-C mutant to accumulate in the nucleus of permeabilized cells. Interestingly, the rate of nuclear entry of the m-C mutant was substantially lower than that of wild-type importin β, but the m-C mutant
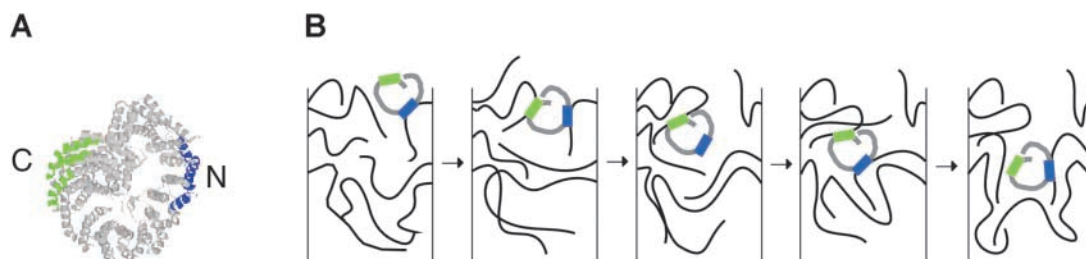
Figure 6.   **Model depicting the involvement of two nucleoporin binding regions in importin β in its translocation through the NPC.** (A) The two nucleoporin binding regions are illustrated in a ribbon representation of importin β. The NH$_2$-terminal region is shown in blue, and the COOH-terminal region in green. (B) One possible model for importin β translocation through the NPC. The model depicts only the central channel region, but movement between the peripheral fibrils of the NPC and the central channel could be conceptually similar. The two nucleoporin binding regions are shown in blue and green as in A, and the FG-rich nucleoporin regions are depicted as flexible filaments. Importin β could use its NH$_2$- and COOH-terminal regions simultaneously or in succession to promote movement through the NPC.

eventually accumulated to similar levels as the wild-type protein (Fig. 5 B). The reduced rate of nuclear entry suggests that the COOH-terminal nucleoporin binding region is important for translocation of importin β through the NPC. Furthermore, the m-C mutant was clearly deficient in nuclear import of BSA-NLS, which was decreased by 45–55% as compared with wild-type importin β, depending on the concentration of importin β in the assay (Fig. 5 C). The twofold decrease in the affinity of m-C for importin α is not predicted to affect the level of nuclear import, as the concentration of importin α in the assay is 390 nM (see Materials and methods).

Examination of the m-N/m-C mutant revealed that it did not accumulate significantly in the nucleus when added to permeabilized cells over the 10-min time course of our assay, indicating that it is highly deficient in translocation through the NPC. Moreover, the m-N/m-C mutant did not support significant nuclear import of BSA-NLS at all receptor concentrations tested (Fig. 5 C), even though its affinity for importin α was only twofold weaker than wild type and the binding to RanGTP was nearly normal (4.4 vs. 3.3 nM for the wild type) (Table II; unpublished data). Considered together, these data demonstrate that both the NH$_2$- and COOH-terminal nucleoporin binding regions of importin β are important for translocation through the NPC in nuclear import.

Discussion

Importin β contains a COOH-terminal nucleoporin binding region in addition to the previously described NH$_2$-terminal region

Biochemical and functional analysis of a large number of importin β mutants allowed us to characterize the interactions between importin β and several FG repeat nucleoporins. Our data indicate that importin β has a second, previously uncharacterized nucleoporin binding region in its COOH-terminal region (in HEAT repeats 14–16), in addition to the NH$_2$-terminal nucleoporin binding area in HEAT repeats 5–7 that was characterized by crystallographic analysis (Bayliss et al., 2000). Interestingly, the two nucleoporin binding areas are located on the opposite side of the protein (Fig. 6 A), and both regions appear to contribute, to a similar extent, to nuclear import (discussed later).

The results of our quantitative nucleoporin binding assays involving a number of point mutants in HEAT repeats 5–7 support the predictions of the crystal structure by Bayliss et al. (2000). However, in our hands, the functional effects of mutations in the single residue that was analyzed previously (I178; Bayliss et al., 2000) were significantly weaker than reported before. Our most severe mutant involving the NH$_2$-terminal nucleoporin binding region that still retained wild-type binding for RanGTP and importin α was the quadruple point mutant m-N. Although the affinity of the m-N mutant for Nup153 was decreased by ∼100-fold, its import activity was decreased by only 35–50%. Our finding that m-N and other importin β mutants having a tremendous decrease in binding affinity for nucleoporins can still support substantial levels of nuclear import indicates that importin β can employ low-affinity interactions to move through the NPC. This is analogous to another nuclear import receptor, transportin, which is estimated to have a K$_d$ of ∼4 μM for nucleoporins (Ribbeck and Görlich, 2001). We identified the COOH-terminal nucleoporin binding region in importin β (located in HEAT repeats 14–16) by using a structural alignment of the NH$_2$- and COOH-terminal halves of importin β, combined with site-directed mutagenesis. This work identified two key residues in the A helices of HEAT repeats 14 and 16 (L612 and F688) that appear to be analogous to two residues of HEAT repeats 5 and 7 (I178 and Y255, respectively), which are important for nucleoporin binding.

We found that a COOH-terminal fragment of importin β (comprising HEAT repeats 8–19) binds saturably to Nup153, although it has a much weaker affinity (∼150-fold) than a fragment containing the NH$_2$-terminal region (HEAT repeats 1–10). Nonetheless, the weak nucleoporin binding is sufficient to mediate the translocation of the COOH-terminal fragment into the nucleus. The mutations L612D and F688A inhibit the binding of the COOH-terminal importin β fragment to nucleoporins, but have no detectable effect on nucleoporin binding when they are introduced into full-length importin β, due to the presence of the high-affinity NH$_2$-terminal region. However, the integrity of the COOH-terminal nucleoporin binding region is crucial for the high-level importin β–mediated nuclear import, as the import activity of an importin β mutation containing both L612D and F688A (the m-C mutant) is reduced by

∼50%. An importin β construct containing both the m-N and the m-C mutations was almost completely inactive in nuclear import, even though it maintained nearly wild-type affinity for RanGTP and for importin α. Considered together, our results indicate that both the NH_2- and COOH-terminal regions of importin β play an important role in binding to FG repeat nucleoporins to direct importin β translocation through the NPC.

In previous work, we found that an NH_2-terminal fragment of importin β containing HEAT repeats 1–11 shows an ∼50% reduction in the nuclear import of PTHrP as compared with full-length importin β, despite the fact that this fragment binds PTHrP NLS and RanGTP with wild-type affinity (Cingolani et al., 2002). In light of the present study, the decrease in the efficiency of PTHrP import with the NH_2-terminal importin β fragment is likely to be due to the absence of the COOH-terminal nucleoporin binding region. This further supports the notion that both the NH_2- and COOH-terminal nucleoporin binding regions of importin β have an important functional role.

Although the structure of the nucleoporin binding site(s) in the COOH-terminal region of importin β is uncertain, by analogy with the structurally homologous NH_2-terminal region, hydrophobic pockets between HEAT repeats 14–15 and 15–16 probably are responsible for FG repeat binding. Because importin β consists entirely of tandem HEAT repeats, it is possible that it contains additional hydrophobic binding pockets in regions adjacent to HEAT repeats 5–7 and 14–16, which can form weak contacts with FG repeats of nucleoporins. In this scenario, the nucleoporin binding regions in HEAT repeats 5–7 and 14–16 would serve as nucleating sites that would allow initial positioning of a nucleoporin FG repeat region on importin β. Upon forming the initial contacts, the interactions could propagate to neighboring HEAT repeats. This might explain the high affinity of importin β for certain nucleoporins, such as Nup153, which is characterized by a high density of FG repeats in its COOH-terminal region (for review see Bednenko et al., 2003).

Two nucleoporin binding sites can enhance translocation of receptors through the NPC

A crucial step in the translocation of cargo–receptor complexes across the NPC involves movement through the central channel, which provides the major barrier to the free diffusion of the macromolecules across the NPC (Feldherr and Akin, 1997). The structure of the central channel is obscure, but it is estimated that the concentration of FG repeat motifs in this region may be very high (50 mM; Bayliss et al., 1999). FG-rich nucleoporin regions have been shown to be flexible and largely unstructured (Denning et al., 2002, 2003), which could allow importin β to contact FG motifs in many nucleoporins in its vicinity as it is translocated through the NPC.

Based on our data, we propose that importin β utilizes its two nucleoporin binding regions coordinately as it translocates through the NPC. A hypothetical view of receptor movement through the diffusionally restricted central channel is illustrated in Fig. 6 B. According to this model, binding of one of the importin β regions to an FG repeat nucleoporin would help to locally tether the receptor and promote its interaction with another nearby nucleoporin. In effect, this could mean that importin β is continuously bound to the NPC as it progresses from one FG repeat nucleoporin to another. It is possible that the two nucleoporin binding regions on importin β communicate with each other, such that the interaction of one binding region with an FG repeat induces a change in importin β conformation and modulates the interaction of the second binding region with the other FG repeat. Alternatively, the activities of the two nucleoporin binding sites may be independent. As the binding of RanGTP to importin β promotes the movement of large cargoes through the central channel (Lyman et al., 2002), it will be interesting to determine if transient RanGTP binding to the NH_2 terminus of importin β selectively weakens FG repeat binding to one of the sites (e.g., the high-affinity, NH_2-terminal site) and not to the other (e.g., the low-affinity, COOH-terminal site).

It is intriguing that two other nuclear transport receptors whose nucleoporin interactions have been analyzed by crystallography, the mRNA nuclear export factor TAP/NXF1 and the Ran import factor NTF2, also contain two nucleoporin binding sites (for review see Bednenko et al., 2003). Similar to importin β, mutations in either nucleoporin binding site of TAP reduce its mRNA export activity, whereas mutations in both sites completely abolish it (Fribourg et al., 2001). In addition, both NH_2- and COOH-terminal fragments of exportin-t, a member of the importin β/karyopherin β family, have been shown to bind to certain nucleoporins and to be able to move through the NPC (Kuersten et al., 2002). It is therefore plausible that the presence of two nucleoporin binding sites is a general feature of nuclear transport receptors.

Structural evolution of importin β

Receptors of the importin β superfamily are proposed to have evolved from an ancient importin, which resembled the NH_2-terminal fragment of importin β (Malik et al., 1997; Cingolani et al., 2002). This model originally was based on a phylogenetic analysis of a number of importin α– and importin β–like transport factors (Malik et al., 1997). It was supported by the observation that the NH_2-terminal fragment of importin β containing HEAT repeats 1–11 can serve as a minimal nuclear import receptor for PTHrP (Cingolani et al., 2002). Our demonstration here of the structural homology and functional overlap between the NH_2-terminal and the COOH-terminal halves of importin β offers further support for this model.

Importin β could have evolved from this prototypical import receptor by obtaining additional HEAT repeats, possibly by gene duplication, which allowed it to broaden its substrate specificity by providing binding sites for adapters such as importin α. In addition, the acquisition of the COOH-terminal nucleoporin binding region by importin β would have increased the efficiency of nuclear import. Structural analysis of complexes between FG repeat nucleoporins and other members of the importin β superfamily should provide further insight into this fundamental question related to nuclear transport receptor evolution.

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Materials and methods

Plasmids

Expression clones for importin β and importin β fragments were constructed by PCR amplification of the 6× his-S–tagged importin β DNA (Chi et al., 1996) and insertion of the PCR products into the NdeI and NotI sites of the pTYB2 vector (New England Biolabs, Inc.). Site-directed mutagenesis of importin β was performed by overlap extension PCR (Ho et al., 1989). All constructs were verified by DNA sequencing.

Protein expression and purification

Wild-type importin β and its point mutants and fragments were expressed as fusion proteins containing COOH-terminal intein and chitin binding domain tags in the *Escherichia coli* ER2566 strain (New England Biolabs, Inc.) under the following conditions. Cultures were inoculated with freshly transformed bacteria and grown at 37°C to an A_{600} of 0.4. Importin β expression was induced by the addition of 0.5 mM IPTG and incubation for 4 h at 22°C. Bacteria were collected and stored at −80°C. For protein purification, cells were resuspended in lysis buffer (50 mM Tris, pH 8.0, 500 mM NaCl, 2 mM $MgCl_2$, 10 mM CHAPS, 10 mM thioglycolic acid, and a protease inhibitor cocktail containing 1 μg/ml each of pepstatin, leupeptin, and aprotinin). The suspension was sonicated three times for 30 s and centrifuged at 100,000 *g* for 30 min. Importin β fusions were purified on chitin beads (New England Biolabs, Inc.) and induced to undergo an intein-mediated self-cleavage and release from the beads by overnight incubation at 4°C in the presence of 30 mM DTT. The purified proteins were dialyzed against transport buffer (20 mM Hepes, pH 7.4, 110 mM KOAc, 2 mM MgOAc, 2 mM DTT, and protease inhibitors) and stored at −80°C. 1 liter of *E. coli* yielded ∼1 mg of >95% pure protein; the importin β mutations we analyzed did not have an effect on the protein yield. All of the importin β mutants used in this study were expressed and purified at least two separate times. Different preparations of the same mutant displayed the same nucleoporin binding properties and nuclear import activity. GST-tagged nucleoporins and transport factors were expressed and purified as described previously (Ben-Efraim and Gerace, 2001), except that a shorter COOH-terminal fragment of Nup153 (residues 895–1475) was used in this study (Nakielny et al., 1999). FITC-labeled BSA-NLS was prepared as described previously (Melchior et al., 1995).

Solid phase binding assay

The solid phase binding assay was performed on microtiter plates (Nunc) essentially as previously described (Delphin et al., 1997). Proteins were adsorbed to microtiter plates by incubating 200 ng of GST-tagged nucleoporin, GST–RanGTP, importin α, or GST with each well. Except as noted below, importin β binding reactions were conducted in PBS containing 0.1% Tween at 4°C for 1.5 h. Importin β was detected using a polyclonal anti–S tag antibody and HRP-coupled secondary antibody. The colorimetric reaction, which was performed using tetramethylbenzidine (Calbiochem), was stopped by the addition of 125 nM HCl, and the signal was measured at 450 nm. The values obtained with GST were subtracted from the values obtained with GST-tagged nucleoporin or GST–RanGTP to correct for nonspecific binding. The apparent K_d values were determined by fitting the data into a binding equilibrium equation using nonlinear regression (KaleidaGraph software). The K_d values varied slightly from experiment to experiment, and variation came mostly from different concentrations of primary and secondary antibodies in different experiments. Therefore, for a given importin β binding partner, we assayed different importin β mutants in the same experiment. We found that the presence of 0.1% Tween in the binding buffer significantly decreased the affinity of the importin β mutants L612D and F688A for importin α. These mutants, however, had wild-type affinity for importin α when assayed in PBS without detergent, PBS containing 30 mg/ml BSA, or PBS containing 30 mg/ml BSA and 0.1% Tween. We concluded that this effect was due to differential binding of Tween to L612D and F688A mutants, which interfered with importin α binding. The L612D and F688A mutations reduced the affinity of the interaction between the COOH-terminal fragment of importin β (304–876) and Nup153 in all the buffers tested.

Nuclear import assay

Analysis of nuclear import in digitonin-permeabilized HeLa cells grown in suspension was performed essentially as described previously (Adam et al., 1992), except that cells were treated with 0.4 μM RanQ69L and 0.8 μM RanBP1 for 15 min at 30°C to deplete endogenous importin β and Ran (Ben-Efraim and Gerace, 2001). Each 40-μl import reaction contained 3 × 10⁵ HeLa cells, 1–3.4 pmol of importin β, 15.6 pmol of importin α, 10 pmol of Ran, 25 pmol of NTF2, 8 pmol of BSA-NLS, and an ATP regeneration system (0.5 mM ATP, 0.8 mg/ml creatine phosphate, 20 U/ml creatine phosphate kinase) in transport buffer. Import reactions were incubated for 30 min at 30°C, and the level of nuclear import was quantified by flow cytometry (Paschal and Gerace, 1995). Control reactions contained 13.8 U/ml hexokinase and 25 μM glucose to deplete ATP. Analysis of nuclear import in adherent cells was performed as described by Bayliss et al. (2000), except that the cells were incubated in transport buffer for 15 min at 30°C before import to deplete endogenous importin β.

Importin β nuclear migration assay

3.4 pmol of importin β in 20 μl was added to digitonin-permeabilized adherent HeLa cells, and the cells were incubated at room temperature for various times. The cells were quickly washed with cold PBS and fixed by addition of 4% formaldehyde in PBS. Importin β was detected by immunofluorescence using anti–S tag polyclonal antibody and FITC-conjugated secondary antibody. The cells were examined by confocal microscopy using the same detection settings for all samples. The level of intranuclear importin β was determined using NIH Image program.

Structural alignment of importin β

Intramolecular structural alignment of importin β was performed using the combinatorial extension (CE) method as implemented at http://cl.sdsc.edu/ce/ce_align.html (Shindyalov and Bourne, 1998). Figures were generated with BOBSCRIPT (Esnouf, 1999) and Rasted3D (Merritt and Bacon, 1997).

Online supplemental material

The supplemental material (Figs. S1–S3) is available at http://www.jcb.org/cgi/content/full/jcb.200303085/DC1. The supplemental material contains confocal images of nuclear import assays with wild-type importin β and importin β mutants (Figs. S1 and S2) as well as with the COOH-terminal importin β fragment (Fig. S3).

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