Molecular Basis for the Recognition of Snurportin 1 by Importin β*S

The nuclear import of uridine-rich ribonucleoproteins is mediated by the transport adaptor snurportin 1 (SNP1). Similar to importin α, SNP1 uses an N-terminal importin β binding (sIBB) domain to recruit the receptor importin β and gain access to the nucleus. In this study, we demonstrate that the sIBB domain has a bipartite nature, which contains two distinct binding determinants for importin β. The first determinant spans residues 25–65 and includes the previously identified importin α IBB (αIBB) region of homology. The second binding determinant encompasses residues 1–24 and resembles region 1011–1035 of the nucleoporin 153 (Nup153). The two binding determinants synergize within the sIBB domain to confer a low nanomolar binding affinity for importin β ($K_d \approx 2 \text{ nM}$) in an interaction that, in vitro, is displaced by RanGTP. We propose that in vivo the synergy of Nup153 and nuclear RanGTP promotes translocation of uridine-rich ribonucleoproteins into the nucleus.

Nuclear import of proteins and nucleic acids is an active signal-mediated process that requires, in most cases, soluble transport factors and GTP hydrolysis by the GTPase Ran (1–4). Nuclear transport factors are sorted into two categories: importins and exportins (also known as karyopherins), which include 14 members in budding yeast and at least 20 in humans (5, 6). All karyopherins fold into superhelical solenoids, which present an external convex surface involved in nucleoporin binding and a concave internal face that interacts with transport cargos and RanGTP. The two best characterized importins, human importin β1 and yeast karyopherin β2, have been visualized using crystallographic methods bound to specific import cargos (7–10) and RanGTP (11, 12). These structures have provided invaluable information on the structural flexibility of karyopherins and their ability to undergo conformational changes during the import process (13–16).

The best understood nuclear import pathway involves cargos bearing a classical SV40-like nuclear localization sequence (PKKKKRKV) (17). These cargos are imported into the nucleus by the adaptor importin α and the receptor importin β with the aid of RanGTP. GTP hydrolysis by Ran energizes the import reaction by promoting both release of the import complex from nucleoporins as well as unloading of import cargos into the nucleus (1–3). In addition to classical nuclear localization sequence cargos, uridine-rich ribonucleoproteins (U snRNP)s represent an important class of import cargos. Mature U snRNP particles are assembled in the cytoplasm and imported into the nucleus in at least two distinct pathways, both dependent on importin β (18). In the first pathway, the import signal is in the proteinaceous core of the U snRNP formed by the Sm proteins (18–21). In the second pathway, the trimethylated guanosine cap of the mature U snRNP is recognized by the adaptor snurportin 1 (SNP1) (18–21), which, in turn, recruits importin β. SNP1 consists of an N-terminal IBB domain (sIBB) similar to that found in importin α (αIBB) and a large C-terminal trimethylated guanosine cap-binding region that resembles the GTP-binding domain of mRNA-guanylyltransferase (22). In permeabilized cells, SNP1 and importin β promote Ran- and energy-independent nuclear import of at least two specific spliceosomal U snRNPs, namely U1 and U5 (23). After nuclear entry, SNP1 is recycled back to the cytoplasm by Crm1 in complex with RanGTP (24). In this study, we have used a combination of crystallographic, biochemical, and biophysical techniques to define the composition and recognition of the sIBB domain by importin β.

EXPERIMENTAL PROCEDURES

Biochemical Techniques—Human importin β1 (residues 1–876) was expressed and purified as described previously in Ref. 8. HEAT repeats 1–10 of importin β (residues 1–445) were cloned into the Ndel and NotI sites of the pTYB2 vector (New England Biolabs, Inc.) and expressed in the Escherichia coli ER2566 strain. Purification of importin β (1–445) was performed as described for the full-length protein in Ref. 25. The IBB constructs sIBB-(1–65), sIBB-(25–65), and αIBB-(11–54) were amplified by PCR from an SNP1 (19) or hSRP1 α (26) template and ligated into a unique NcoI site of the pGEX-4T vector. The construct sn-(1–24) was generated by introducing a stop

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The atomic coordinates and structure factors (code 2P8Q, 2Q5D) have been deposited in the Protein Data Bank, Research Collaboratory for Structural Bioinformatics, Rutgers University, New Brunswick, NJ (http://www.rcsb.org/).

The on-line version of this article (available at http://www.jbc.org/) contains two supplemental figures.

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2 The abbreviations used are: U snRNP, uridine-rich small nuclear ribonucleoprotein; SNP1, snurportin 1; IBB, importin β binding; sIBB, SNP1 IBB; αIBB, importin α; NPC, nuclear pore complex; Nup153, nucleoporin 153; ITC, isothermal titration calorimetry; SPR, surface plasmon resonance; GST, glutathione S-transferase.
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codon at position 25 of the plasmid pGEX-sIBB-(1–65), GST-sIBBs and GST-sIBB constructs were expressed in *E. coli* BL21 (DE3) strain for 3 h at 30 °C after induction with 0.5 mM isopropyl-1-thio-β-D-galactopyranoside and purified over GST beads followed by gel filtration chromatography. Ran was expressed, purified, and loaded with the non-hydrolyzable GTP analog, GppNHP, as described previously (27).

**Crystallization and Structure Determination**—Importin β bound to a chemically synthesized sIBB-(25–65) peptide was crystallized under 20% polyethylene glycol 8000, 50 mM sodium chloride, at pH 6.0. Crystals of the importin β-sIBB-(25–65) complex diffracted weakly to 5 Å resolution. Prolonged dehydration of crystals by soaking in 38% polyethylene glycol 8000 dramatically improved the diffraction to 2.35 Å resolution. Approximately 50 crystals were soaked for various times and screened at beamline X6A at the Brookhaven National Light Source (BNLS) on a Quantum Q4 CCD detector. Diffraction data were reduced to intensities using the programs DENZO and SCALEPACK (28) (see Table 1). Two distinct crystal forms were obtained, both in space group P2₁. Crystal form I (see ds2.35 in Table 1) contains one importin β-sIBB-(25–65) complex in the asymmetric unit. Crystal form II (see ds3.2 in Table 1), which diffracted weakly to 3.2 Å resolution, contains two importin β-sIBB-(25–65) complexes in different conformations per asymmetric unit. Both structures were solved by molecular replacement in MolRep (29) using human importin β (Protein Data Bank (PDB) code 1QGK) as a search model. The initial solution was refined in CNS (30) using rigid body refinement, simulated annealing, and grouped B-factor refinement to an $R_{free}$ ~ 35% (calculated using 10% of the observed reflections). The sIBB-(25–65) domain was built in $F_o - F_c$ electron density difference maps using the program Coot (31). After several rounds of manual building alternated with positional and B-factor refinement, the final model for crystal form I has a $R_{work}$ and $R_{free}$ of 22.7 and 25.0%, respectively. For crystal form II, the final model was refined to a $R_{work}$ of 30.3% and $R_{free}$ of 32.8%, using all reflections between 40 and 3.2 Å resolution and includes a well defined closed importin β-sIBB domain complex (complex A) and an open conformation of the complex (complex B). All structural figures were made using PyMOL (32).

**Native Gel Electrophoresis and Pull-down Assay**—Native binding assay on agarose gel was carried out as described in Ref. 33. In the assay, 25 μg of importin β, a gel filtration-purified complex of importin β bound to either sIBB-(1–65) or sIBB-(25–65) peptides, were separated on a 1.5% agarose gel at 30 °C followed by extensive washing in pull-down buffer (20 mM HEPES, pH 7.4, 150 mM sodium chloride, 3 mM β-mercaptoethanol, 0.005% Tween 20). After washing, all samples were dissolved in SDS-loading buffer and analyzed on SDS-PAGE.

**Isothermal Titration Calorimetry (ITC)**—ITC experiments were carried out at 30 °C in a ITC calorimeter (Microcal). sn-(1–24) dissolved in β-buffer (10 mM HEPES, pH 7.4, 150 mM sodium chloride, 3 mM EDTA, 3 mM β-mercaptoethanol) at a concentration of 450 μM was injected in 9-μl increments into the calorimetric cell containing 1.8 ml of importin β in β-buffer at a concentration of 60 μM. The spacing between injections was 360 s. Titration data were analyzed using the Origin 7.0 data analysis software (Microcal Software, Northampton, MA). Injections were integrated following manual adjustment of the baselines. Heats of dilution were determined from control experiments with the β-buffer and subtracted prior to curve fitting using a single set of binding sites model. The curve fitting yields a $K_f = 30 ± 14 μM$ and $ΔH = 6948 ± 1491$ cal/mol at 30 °C for titration of the sn-(1–24) peptide into full-length importin β and a $K_d = 30 ± 15 μM$ and $ΔH = 6548 ± 1191$ cal/mol for titration of the sn-(1–24) peptide into importin β (residues 1–445).

**Surface Plasmon Resonance (SPR)**—GST control, GST-sIBB-(25–65), GST-sIBB-(1–65), and GST-sIBB-(11–54) were captured onto a sensor chip using an immobilized anti-GST antibody. Importin β was flowed into the cell in β-buffer (plus 0.005% surfactant P20) at concentrations between 200 and 600 nm, at a flow rate of 60 μl/min. Data were analyzed using the BIAevaluation software (Biacore Life Sciences). The association and dissociation curves were fit separately using the simple 1:1 Langmuirian model. The analysis of each individual curve resulted in an apparent rate of association and dissociation $k_{on} = 7.22 e^4 ± 1.8 e^4$ (1/ms), $k_{off} = 8.24 e^4 ± 0.5 e^{-4}$ (s⁻¹), and $k_{on} = 1.01 e^5 ± 0.3 e^4$ (1/ms), $k_{off} = 2.07 e^{-4} ± 0.3 e^{-4}$ (s⁻¹) for sIBB-(25–65) and sIBB-(1–65), respectively. The dissociation constants of the sIBB-(25–65) and sIBB-(1–65) for importin β measured as $K_d = k_{off}/k_{on}$ was $K_d = 15.6 ± 5.8$ nm and $K_d = 20.4 ± 0.4$ nm, respectively.

**RESULTS**

**Snurportin IBB Domain Is Bipartite**—Nuclear import SNP1 is mediated by the N-terminal sIBB domain (residues 1–65) (19). This region alone is necessary and sufficient to promote nuclear import in the absence of Ran and energy in a permeabilized cell assay (23). Analysis of the primary sequence of the sIBB domain suggests the presence of a bipartite signal (Fig. 1a). Region 25–65 of the sIBB domain closely resembles the classical IBB domain of importin α (sIBB), which promotes Ran/energy-dependent nuclear import (23, 34). Similar to the sIBB domain (residues 11–54), all basic residues critical for importin β binding are conserved in the sIBB-(25–65) domain (supplemental Fig. 1). The latter is, however, shorter due to a 3-amino-acid gap between residues 46 and 47 (Fig. 1a). The region 25–65 of the sIBB domain will be defined as the αIBB region of homology. In contrast to importin α, which show poor sequence conservation upstream of the IBB domain, SNP1 residues 1–24, sn-(1–24), are well conserved in snurportins (supplemental Fig. 1). Blast analysis reveals that sn-(1–24) shares 42% sequence identity and 79% similarity to a region of Nup153 spanning

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residues 1011–1035. This same region of Nup153 binds importin β with high affinity, likely via interactions with phenylalanine-glycine (FXFG) repeats (25).

Structure of Importin β Bound to the sIBB-(25–65) Domain—To shed light on the interaction of SNP1 with importin β, we crystallized human importin β bound to a chemically synthesized peptide spanning residues 25–65 of SNP1. Although crystallization failed for this same region of Nup153, we observed a similar sequence of events:

1. Using the method of molecular replacement, we determined the structure of the sIBB-(25–65) domain in the asymmetric unit of the complex (in green) bound to the sIBB-(25–65) domain.

2. Aiming at high resolution diffraction studies, we subjected the importin β-sIBB-(25–65) complex crystals to dehydration in highly concentrated solutions of polyethylene glycol 8000. Diffraction analysis confirmed a dramatic improvement in the diffraction quality and resolution limit as a function of the time of dehydration. Dehydration for 3–12 h yielded high quality diffraction past 2.3 Å resolution. This crystal form, referred to as crystal form I, displayed a small primitive monoclinic unit cell (Table 1), which contained one importin β-sIBB-(25–65) complex in the asymmetric unit and ~55% solvent content.

The structure of crystal form I was determined by the molecular replacement method and refined to an R free of 25.0%, including all reflections between 40 and 2.35 Å resolution (Fig. 1b and Table 1). In the structure, importin β adopts a closed conformation nearly identical to that seen in complex with the αIBB domain (8). The protein is built by 19 HEAT repeats, which wrap around the sIBB-(25–65) in a conformation reminiscent of a snail.

The sIBB-(25–65) domain interacts with the concave surface of importin β between HEAT repeats 7–19. The sIBB-(25–65) domain folds as a

FIGURE 1. Structure of the sIBB domain. a, the sIBB domain spans residues 1–65 of SNP1 and contains two structural determinants: region 1–24, sn-(1–24), has 42% identity to region 1011–1035 of Nup153, whereas region 25–65 is 55% identical to the IBB domain of importin α1 (hSRP1α) (26). In the alignment, identical residues are colored in red, b, left panel, ribbon diagram of crystal form I of importin β (in green) bound to the sIBB-(25–65) domain (in magenta), determined at 2.35 Å resolution. Right panel, blowup of the sIBB-(25–65) domain.

### TABLE 1

X-ray data collection and refinement statistics for importin β-sIBB-(25–65) complexes

| Data collection statistics | ds2.35 (2P8Q) | ds3.2 (2Q5D) |
|----------------------------|--------------|--------------|
| Unit cell dimensions (Å) | a = 64.75, b = 97.89, c = 84.28 | a = 102.9, b = 100.7, c = 108.7 |
| B value from Wilson plot (Å²) | 40.30 | 40.30 |
| Total observations | 227,438 | 50,424 |
| Unique observations | 44,104 | 27,142 |
| Completeness (%) | 95.7 (64.1) | 78.3 (51.7) |
| Rsym (%) | 8.2 (47.0) | 108.4 (49.0) |
| (I/σ(I)) | 20.1 (2.6) | 8.14 (1.1) |

| Refinement statistics | ds2.35 (2P8Q) | ds3.2 (2Q5D) |
|----------------------|--------------|--------------|
| Number of reflections | 43,296 | 31,762 |
| Rwork, Rfree (%) | 22.7/25.0 | 30.3/32.8 |
| Number of water molecules | 345 | 345 |
| B value of model (Å²) | 47.7 | 83.5 |
| r.m.s. deviation from ideal bond length (Å) | 0.008 | 0.010 |
| r.m.s. deviation from ideal bond angles (degree) | 1.0 | 1.4 |
| Ramachandran plot (%) | Core region: 92.0 | 85.3 |
| Allowed region | 7.6 | 14.2 |
| Generously allowed region | 0.4 | 0.4 |
| Disallowed region | 0.1 | 0.2 |

* $R_{sym} = \sum_i |I_i(h)| - |\langle I(h)\rangle|/\sum_i |I_i(h)|$ where $I_i(h)$ and $\langle I(h)\rangle$ are the ith and mean measurement of intensity of reflection h.

* The $R_{free}$ value was calculated using 10% of the data.
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FIGURE 2. Structural plasticity of importin β bound to the sIBB-(25–65) domain. a, ribbon diagram of the asymmetric unit content of crystal form II, determined at 3.2 Å resolution. The two complexes in the asymmetric unit have different conformations. In complex B, on the left (in cyan), importin β adopts an open conformation, and only the sIBB-(40–65) helix is visible (in yellow). In complex A, on the right (in green), importin β has a conformation identical to crystal form I, and all residues for the sIBB-(25–65) domain are visible (in magenta). b, superimposition of the importin β structures from complex A (closed) and B (open) reveals deviations up to 20 Å in the C terminus of the protein.

long C-terminal helix (residues 41–65), connected to an N-terminal 3₁₀ helix (residues 27–30) by a 7-residue spoon-shaped linker (Fig. 1b). The overall B-factor for the peptide is ~65 Å², slightly higher than that for the importin β (~45 Å²).

Crystal form II was obtained upon short dehydration (~30 min) of importin β-sIBB-(25–65) crystals and diffracted to ~3.2 Å resolution. Interestingly, this second crystal form contains two importin β-sIBB-(25–65) complexes in different conformations per asymmetric unit (Fig. 2a). In one complex (referred to as complex A), importin β adopts a closed conformation nearly identical to crystal form I. In the second complex (complex B), importin β is distinctly open, with C-terminal HEAT repeats 12–19 swung up to 20 Å away from the corresponding position in the closed conformation (Fig. 2b). The tertiary structure of the importin β molecule in complex B resembles Kap95p, the yeast homologue of importin β, in complex with RanGTP (11). This open conformation of importin β likely represents the structure adopted by the protein in the process to unload the import cargo. Accordingly, the electron density for the sIBB-(25–65) domain is less defined in the complex B, where only the C-terminal helix is visible.

sIBB-(25–65) versus αIBB Domain—The structure of importin β bound to the αIBB-(11–54) domain was previously determined to a comparable resolution of 2.3 Å (8), allowing for direct comparison with the crystal form I described in this study. Despite the profound functional differences between the sIBB and αIBB domain, the conformation adopted by importin β in the two complexes is virtually identical (r.m.s. deviation 0.965 Å) (Fig. 3a). It is noteworthy that significant differences exist in the structure of the two IBB domains and in the way importin β positions them inside the cargo-binding surface (Fig. 3a). First, the sIBB-(25–65) domain is slightly shorter than the αIBB domain (35 versus 41 Å), which is consistent with the 3-amino-acid gap in the C-terminal helix between residues 46 and 47 (Fig. 1a). Second, the sIBB C-terminal helix is ~5° tilted with respect to the αIBB helical axis, which positions it closer to the importin β concave surface (Fig. 3a). Third, in the sIBB-(25–65) domain, the linker between the C-terminal helix and the N-terminal 3₁₀ helix is significantly longer than in the αIBB domain (7 versus 3 amino acids), which suggests in the sIBB domain that these two helices have a much higher degree of flexibility. This hypothesis is corroborated by the structural plasticity seen in the 3.2 Å crystal form II, where in the more open complex B (Fig. 2a) only the C-terminal helix of the sIBB domain is visible, whereas the N-terminal 3₁₀ helix is likely flexible and thus disordered in the crystal structure.

As in the recognition of the αIBB-(11–54) domain, importin β makes two distinct sets of contacts with the sIBB domain. The first includes the N-terminal moiety of the sIBB peptide (residues 25–40), which interacts with HEAT repeats 7–11 (Fig. 3b). This region is critical for binding of SNP1 to importin β. Substitution of Arg ²⁷ to Ala decreases the affinity of SNP1 for importin β by 20-fold and blocks nuclear import in digitonin-permeabilized cells (35). The second region of interaction involves the sIBB helix (residues 41–65), which presents an extended network of contacts with HEAT repeats 12–19 of importin β (Fig. 3b). In the sIBB domain, this region engages in fewer contacts, 12 versus 20, as compared with the αIBB helix (residues 26–54). Likewise, the polybasic stretch ²⁷Arg-Arg-Arg-Arg ¹¹ of the αIBB domain is shifted in the sIBB helix where only 2 of the 4 arginines contact importin β directly (Fig. 3b). SNP1 Region (1–24) Contains a Binding Determinant for Importin β—We next sought to determine whether the Nup153-like moiety of SNP1, sn-(1–24), binds importin β directly. The interaction of the sn-(1–24) with importin β was probed using three independent binding techniques. First, by native electrophoresis on agarose gel, a chemically synthesized sn-(1–24) peptide was found to shift the migration of importin β, which is suggestive of a direct physical interaction between the peptide and the protein (Fig. 4a). Second, using ITC, titration of sn-(1–24) in a calorimetric cell containing importin β at 30 °C yielded an endothermic binding reaction that is adequately described by a single exponential decay binding model (Fig. 4b). The thermodynamic parameters obtained from the curve fit indicated that sn-(1–24) binds importin β with a Kd ~ 30 ± 14 μM and an enthalpy of complex formation of ΔH = 6948 ± 1491 cal/mol. Interestingly, identical heat release (and thus Kd) was observed using a fragment of importin β comprising HEAT repeats 1–10 (residues 1–445) (Fig. 4c). These evidences lend support to the existence of a single binding site for the sn-(1–24) in the N-terminal, RanGTP- (11, 27), and nucleo-
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shorter sIBB-(25–65) domain has a 7-fold higher binding affinity for importin β than the shorter sIBB-(25–65) domain lacking the sn-(1–24) (Kd = 2.0 ± 0.5 nM versus 15.2 ± 5.8 nM) (Fig. 5). The affinity measured by SPR for the full-length sIBB-(1–65) domain agrees well with the Kd values measured by FRET (Kd = 2.0 nM) (37) and solid phase binding assay (Kd = 4.7 nM) (35), which makes us confident in the accuracy of this technique in measuring the interactions of importin β with the SNP1. In addition to a significant drop in the Kd, the sIBB-(25–65) domain had a 4-fold faster rate of dissociation from importin β as compared with the sIBB-(1–65) domain (kdoff = 8.24 ± 0.5 e3 s⁻¹ versus koff = 2.07 ± 0.3 e3 s⁻¹) (Fig. 5), whose off rate from importin β was comparable with that of importin α (38).

RanGppNHp Displaces the sIBB Domain from Importin β—We next investigated whether the interaction between SNP1 and importin β is disrupted by the small GTPase Ran, in vitro. By pull-down assay, agarose beads coupled to GST-sIBB-(1–65) efficiently pulled-down importin β (Fig. 6, lane 4). This interaction was selectively disrupted by an excess of RanGppNHp as compared with RanGDP (Fig. 6, lanes 5 and 6, respectively). In a second set of experiments, we sought to determine whether the N-terminal moiety sn-(1–24) alters the affinity of Ran for the importin β-sIBB complex. To address this question, we isolated on a Superdex 200 gel filtration column a 1:1 complex of importin β bound to either the sIBB-(1–65) or the sIBB-(25–65) domain. The stoichiometric presence of sIBB peptides was confirmed on Tricine gel (data not shown). Increasing quantities of Ran preloaded with non-hydrolyzable GTP analog, RanGppNHp, were titrated into the preformed gel filtration-purified importin β-sIBB-(25–65) or importin β-sIBB-(1–65) complex, and the mixture was analyzed by native electrophoresis on agarose gel (Fig. 7). In agreement with the pull-down assay, RanGppNHp selectively dissociated both sIBB-(25–65) and sIBB-(1–65) domains from importin β as compared with RanGDP (supplemental Fig. 2). Although both peptides were dissociated by approximately equimolar quantities of RanGppNHp, the dissociation of the domain lacking the sn-(1–24) appeared reproducibly less cooperative than the sIBB-(1–65) domain (in Fig. 7, a versus b, compare lanes 4–8). This may suggest that importin β becomes more sensitive to RanGppNHp binding when the SNP1 moiety sn-(1–24) is bound to its N-terminal domain.

DISCUSSION

To determine the molecular basis for the sIBB-(1–65) karyopheric properties, we have dissected the structure of the sIBB-(1–65) domain and its binding interactions with importin β. The main conclusion of our work indicates that the sIBB-(1–65) domain has a bipartite organization, which consists of two moieties, both interacting with importin β. The first binding determinant of SNP1 spans residues 25–65 and includes an αIBB region of homology, which binds importin β with nanomolar affinity (Kd ~ 15 nM). As shown in the crystal structure of the importin β bound to the sIBB-(25–65) domain, this region of SNP1 interacts with HEAT repeats 7–19 of importin β similar to an αIBB domain and not only with the N terminus of the protein (residues 1–618), as reported previously (20). Within the sIBB-(25–65) domain, the C-terminal helix (residues 41–65) makes fewer and weaker contacts with importin β than the N-terminal residues 25–40 of SNP1. This observation partially explains why an N-terminal fragment of importin β (residues 1–618) shows relatively high binding affinity for the sIBB...
domain (20, 35), but it fails to bind the IBB domain (39). Likewise, the paucity of contacts between the sIBB helix (residues 41–65) and importin β may explain the fast rate of dissociation measured by SPR between importin β and the sIBB-(25–65) domain as compared with its counterpart, the IBB domain (38).

In addition to the IBB region of homology, SNP1 presents a novel binding determinant for importin β between residues 1 and 24. This region interacts weakly with importin β ($K_d = 30 \pm 14 \text{ M}$) when isolated from SNP1 but synergizes with the sIBB-(25–65) moiety in the context of the sIBB-(1–65) domain to confer low nanomolar binding affinity and a slow rate of dissociation from importin β. Therefore, we propose that the sn-(1–24) acts as a molecular hook to retain SNP1 bound to importin β. Interestingly, sn-(1–24) shares high sequence identity to the region 1011–1035 of Nup153, which is localized at the nuclear basket (40) and is known to bind importin β with nanomolar affinity (25). This suggests the intriguing idea of a direct displacement of the sn-(1–24) from importin β within the snRNP-SNP1-importin β import complex at the nuclear basket.

Notably, this scenario is conceptually analogous to what was reported for the yeast nucleoporin Nup2p, which is also associated with the nuclear basket (41, 42). Residues 1–51 of Nup2p bind tightly to Kap60p, the yeast homologue of importin α, and thus accelerate the release of nuclear localization sequence cargos from Kap60p. In addition, the region 1–24 of SNP1 was also shown to be critical for binding to Crm1, which, in complex with RanGTP, recycles SNP1 back to the cytoplasm (24).

How do our data fit with the observation that in digitonin-permeabilized cells, the sIBB domain promotes Ran- and energy-independent nuclear import (23)? The structural and biochemical work presented in this study challenges the idea of a simple Ran-independent nuclear import pathway in at least three ways. First, at the three-dimensional level, the conformation of importin β bound to the sIBB domain is very similar to that adopted in complex with the IBB domain, which requires RanGTP to undergo a dramatic conformational change necessary to release the import cargo (11). Second, the sIBB domain binds importin β with low nanomolar binding affinity, which is incompatible with a Ran-independent cargo release into the nucleus. Third, in vitro, RanGTP, and not RanGDP, specifically disrupts the importin β-sIBB complex. To complement these data, we provide evidence that region 1–24 of SNP1 contains a Nup153-like motif that modulates both the strength of interaction and the off rate of the sIBB domain from importin β. We propose that the sn-(1–24) plays an important functional role during the import reaction by reducing the avidity of the NPC for the sIBB-import complex. This could be explained, for instance, by a simple intermolecular contact between the SNP1 moiety sn-(1–24), which contains a $^{12}$FSVS$^{15}$ repeat, and the major nucleoporin-binding site in importin β (25). Such an interaction would reduce the avidity of the sIBB-

FIGURE 4. sn-(1–24) contains a binding determinant for importin β. a, native electrophoresis on agarose gel. Lane 1, free importin β; lane 2, importin β in the presence of a 3-fold excess of sn-(1–24) peptide. b and c, ITC analysis of the interaction of the sn-(1–24) peptide with importin β. ITC titration of the sn-(1–24) peptide into a calorimetric cell containing either full-length importin β (residues 1–876) (b) or a C-terminally truncated fragment of importin β spanning residues 1–445 (c) was performed. In both b and c, the raw data are in the top panel, and the integrated enthalpy plotted as a function of the sn-(1–24):importin β molar ratio is shown in the bottom panel. The dissociation constants measured from the ITC data for the interactions sn-(1–24):importin β and sn-(1–24):importin β-(1–445) are $K_d = 30 \pm 14 \text{ M}$ and $K_d = 30 \pm 15 \text{ M}$, respectively.

β. Interestingly, sn-(1–24) shares high sequence identity to the region 1011–1035 of Nup153, which is localized at the nuclear basket (40) and is known to bind importin β with nanomolar affinity (25). This prompts the intriguing idea of a direct displacement of the sn-(1–24) from importin β within the snRNP-SNP1-importin β import complex at the nuclear basket. Notably, this scenario is conceptually analogous to what was reported for the yeast nucleoporin Nup2p, which is also associated with the nuclear basket (41, 42). Residues 1–51 of Nup2p bind tightly to Kap60p, the yeast homologue of importin α, and thus accelerate the release of nuclear localization sequence cargos from Kap60p. In addition, the region 1–24 of SNP1 was also shown to be critical for binding to Crm1, which, in complex with RanGTP, recycles SNP1 back to the cytoplasm (24).

How do our data fit with the observation that in digitonin-permeabilized cells, the sIBB domain promotes Ran- and energy-independent nuclear import (23)? The structural and biochemical work presented in this study challenges the idea of a simple Ran-independent nuclear import pathway in at least three ways. First, at the three-dimensional level, the conformation of importin β bound to the sIBB domain is very similar to that adopted in complex with the IBB domain, which requires RanGTP to undergo a dramatic conformational change necessary to release the import cargo (11). Second, the sIBB domain binds importin β with low nanomolar binding affinity, which is incompatible with a Ran-independent cargo release into the nucleus. Third, in vitro, RanGTP, and not RanGDP, specifically disrupts the importin β-sIBB complex. To complement these data, we provide evidence that region 1–24 of SNP1 contains a Nup153-like motif that modulates both the strength of interaction and the off rate of the sIBB domain from importin β. We propose that the sn-(1–24) plays an important functional role during the import reaction by reducing the avidity of the NPC for the sIBB-import complex. This could be explained, for instance, by a simple intermolecular contact between the SNP1 moiety sn-(1–24), which contains a $^{12}$FSVS$^{15}$ repeat, and the major nucleoporin-binding site in importin β (25). Such an interaction would reduce the avidity of the sIBB-
import complex for nucleoporins and possibly allow translocation of the import complex through the NPC in permeabilized cells, in the absence of exogenous RanGTP. The idea of a reduced avidity of the NPC for the sIBB-import complex agrees well with the observation that a chimera of the sIBB-(1–65) fused to the /H9252-galactosidase, sIBB-/H9252-galactosidase, remains associated for less time to the nuclear basket than an /H9251-IBB-/H9252-galactosidase, suggesting a reduced stalling of the sIBB-import complex inside the NPC (20).

However, if the reduced avidity of the sIBB-import complex for the NPC may be sufficient to explain why in permeabilized cells the sIBB domain translocates efficiently without the addition of exogenous Ran, it does not explain in an in vivo setting what energizes the active release (and thus nuclear import) of sIBB-import cargos into the cell nucleus. In agreement with the observation that in vitro the sIBB-import /H9253 interaction is specifically disrupted by RanGTP, we propose that GTP hydrolysis by the small GTPase Ran remains the driving force of the dissociation of SNP1 from importin /H9252. Further studies are needed to better understand this important import pathway. In particular, it will be necessary to develop a functional assay that allows discrimination between nuclear passage of the sIBB-import complex and dissociation from importin /H9252, and that defines the energetic requirement of these two processes.

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