Importin $\beta$ mediates active passage of cellular substrates through the nuclear pore complex (NPC). Adaptors such as importin $\alpha$ and snurportin associate with importin $\beta$ via an importin $\beta$ binding (IBB) domain. The intrinsic structural flexibility of importin $\beta$ allows its concerted interactions with IBB domains, phenylalanine-glycine nucleoporins, and the GTPase Ran during transport. In this paper, we provide evidence that the nature of the IBB domain modulates the affinity of the import complex for the NPC. In permeabilized cells, importin $\beta$ imports a cargo fused to the snurportin IBB (sIBB) with $\sim70\%$ reduced energy requirement as compared with the classical importin $\alpha$ IBB. At the molecular level, this is explained by an $\sim200$-fold reduced affinity of importin $\beta$ for Nup62, when bound to the sIBB. Consistently, in vivo, the importin $\beta$sIBB complex has greatly reduced persistence inside the central channel of the NPC. We propose that by controlling the degree of strain in the tertiary structure of importin $\beta$, the IBB domain modulates the affinity of the import complex for nucleoporins, thus dictating its persistence inside the NPC.

The passage of most cellular substrates through the NPC is an active, signal-mediated process that requires soluble transport factors and the GTPase Ran (1, 2). The largest family of transport factors known, the importin-$\beta$-superfamily (also known as $\beta$-karyopherins), includes at least 20 different known importins and exportins in humans (3). All $\beta$-karyopherins fold into superhelical solenoids composed of $18-21$ tandemly repeated HEAT repeats (4–6). The structures of human importin $\beta$1 and yeast karyopherin $\beta$2 bound to specific import cargos (7–11) and RanGTP (12, 13) have been determined crystallographically. Recently, atomic models have been reported for the prototypical export factor CRM1 (14, 15), which mediates export of nuclear export signal bearing cargos, and for two specific export factors, Cse1 (16, 17) and Xpot (18), dedicated to export importin $\alpha$ and tRNA, respectively. From this structural repertoire, it appears that all $\beta$-karyopherins present an outer surface exposing FG repeat-binding pockets and an inner face, which binds transport cargos and RanGTP. Furthermore, all $\beta$-karyopherins are highly flexible and undergo dramatic conformational changes upon binding to transport cargos and RanGTP. This has been demonstrated using biochemical and biophysical techniques in solution (19–21), in crystal (6, 18), and in silico (22, 23). The united view of $\beta$-karyopherins describes these proteins as flexible solenoids that shuttle between a cytoplasmic and a nuclear conformation, in relation to cargo and Ran binding (4–6).

GTP hydrolysis by the small GTPase Ran is the driving force of nuclear transport. Ran releases the import complex from high affinity binding sites in the NPC and unloads import cargos into the cell nucleus (1, 2). In addition, Ran controls the directionality of transport through its natural concentration gradient of a nuclear RanGTP pool and cytoplasmic Ran being predominately in the RanGDP form. If the involvement of Ran in nuclear transport is widely accepted, the actual mechanism by which karyopherins translocate through the NPC bound to their import/export substrates remains poorly understood. The importance of a physical interaction between importin $\beta$ and the FG repeats found in nups during nuclear transport has been demonstrated (24–26). It was proposed that importin $\beta$ moves through the NPC following a gradient in affinity for FG nups (27). However in yeast, removal or swapping of large FG regions was not found to disrupt nucleocytoplasmic trafficking across the NPC (28). This suggests that the interaction of importin $\beta$ with FG nups can facilitate transport but does not determine the directionality of movement through the NPC (29). Directionality, instead, is imposed solely by the Ran-mediated hydrolysis of GTP, which takes place upon dissociation of cargos from import/export receptors (30).

Uridine-rich ribonucleoproteins (U snRNPs) are large import cargos (e.g. U5 snRNP has a molecular mass of $>1$ MDa (31)) that are assembled in the cytoplasm of eukaryotic cells and imported into the nucleus in two distinct pathways, both dependent on importin $\beta$ (32–35). In the first pathway, the proteinaceous core of the U snRNP formed by the Sm proteins exposes a poorly characterized nuclear localization signal that is recognized by importin $\beta$ (32–35). In the second pathway, a...
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specific import adaptor, SNP1, binds the trimethylated guanosine cap of the mature U snRNP and promotes entry into the cell nucleus in complex with importin α (32–35). SNP1 is functionally analogous to the classical adaptor importin α, which recognizes nuclear localization signal-bearing cargos (36). Similar to importin α, SNP1 contains an N-terminal IBB domain (sIBB), whereas the rest of the protein folds into an trimethylated guanosine cap-binding domain that resembles the GTP-binding domain of mRNA-panynlytransferase but has no significant sequence or structural similarity to importin α (37). The sIBB domain encompasses residues 1–65 (32) and presents a bipartite structure (10). SNP1 residues 1–24, referred to as sn(1–24), has sequence homology to Nup153 and contains a functional nuclear export signal for SNP1 (14, 15), which in vitro binds importin β with a $K_d$ value of $\sim 30 \mu M$ (10). In contrast, sIBB(25–65) contains an αIBB-like region of homology that binds importin α with nanomolar affinity (10).

Mounting evidence has suggested the nuclear import of spliceosomal U snRNPs bound to the adaptor SNP1, and importin β is functionally different from the import of classical nuclear localization signal-bearing cargos, which use a heterodimer of importin α/β. Huber et al. (31) reported that in digitonin-permeabilized HeLa cells, nuclear import of spliceosomal U snRNPs U1 and U5 proceeds efficiently using recombinant SNP1 and importin β in the absence of Ran and GTP hydrolysis. The unusual karyophilic properties of SNP1 were found to be solely dependent on the nature of the sIBB domain (residues 1–65), because a chimera of β-galactosidase fused to the sIBB domain was also imported without either Ran or GTP (31).

Considering the large size of snRNPs, the finding of Huber et al. conflicts with the observation that in the importin α/β import pathway, efficient transport of large proteins in permeabilized cells is critically dependent on GTP and the small GTPase Ran (38). Likewise, Rollenhagen et al. (33) found that although both use importin β, adaptors SNP1 and importin α mediate import of their respective cargos via distinct import pathways. In addition, the sIBB-import complex was found to be released at the nuclear basket faster than the αIBB complex, suggesting a different mechanism of cargo release into the cell nucleus (33). In the present study, we have analyzed the mechanisms by which importin β bound to the sIBB domain enters the cell nucleus, as compared with the classical importin α/β complex. We found that the structure of the IBB C-terminal helix affects the energetic requirement for nuclear import as well as the persistence of the import complex inside the central channel of the NPC by modulating the affinity of importin β for Nup62.

EXPERIMENTAL PROCEDURES

Biochemical Techniques—Importin β (10), importin α (39), Ran (10), RanBP1 (40), and NTF2 (41) were expressed in Escherichia coli and purified as previously described. Δ44-importin β was cloned by long PCR by removing the first 44 residues from the importin β gene and purified as the full-length protein. mCherry (mCh)-importin β was generated by inserting the mCh gene upstream of the importin β gene cloned in pQE60 vector (Qiagen). mCh-importin β was expressed in M15 cells and purified by metal chelate affinity chromatography over nickel-nitrilotriacetic acid resin and anion exchange on a monochromatography column (GE Healthcare). IBB constructs sIBB(1–65), sIBB(25–65), and αIBB(11–54) were ligated into a unique NcoI site of the pGEX-4T vector (GE Healthcare), which also contains a PreScission Protease cleavage site engineered between the GST and the IBB genes. sIBB(25–3A-65) was constructed by long PCR on the sIBB(25–65) template. GST-IBBs were expressed and purified as described (10). To form homogeneous complexes of importin β bound to different IBB domains, each IBB construct was expressed as a GST fusion, immobilized on glutathione beads (GE Healthcare), and washed extensively in a low salt buffer (20 mM HEPES, pH 7.5, 50 mM NaCl, 10 mM β-mercaptoethanol, and 1 mM phenylmethylsulfonyl fluoride). Full-length importin β or mCh-importin β was allowed to incubate with the immobilized IBB domain. PreScission protease was added to cleave the importin β/IBB complexes from the GST. Importin β/IBB complexes were further purified by gel filtration chromatography on a Superdex 200 column (GE Healthcare) and concentrated, and stocks were frozen and stored at $-80^\circ$C. GST-GFP-IBB cargos were generated by inserting the GFP gene in the pGEX4T-IBB plasmid, between the GST and IBB genes. GST-GFP-IBBs were expressed overnight at 22 °C in BL21 E. coli strain, purified on glutathione beads, and eluted with 20 mM glutathione. This elution contained $\sim 50\%$ GST-GFP-IBB fusion proteins contaminated with GST and GST-GFP degradation products. GST-GFP-IBB fusion proteins were further purified over an importin β affinity column obtained by covalently coupling importin β to CNBr-Sepharose beads (GE Healthcare). GST-GFP-IBB fusion proteins were eluted with 150 mM magnesium chloride and gel-filtrated into transport buffer. Expression and purification of FG-rich domains of Nup358 (residues 2503–2893) (27), Nup214 (residues 1918–2090) (42), Nup153 (residues 946–1472) (27), and full-length Nup62 (27) were previously described.

Nuclear Import Assay—Nuclear import assay in digitonin-permeabilized cells was carried out as previously described (43–46). Briefly, HeLa cells were grown to $\sim 50\%$ confluence on coverslips and permeabilized with 20 μg/ml digitonin for 5 min at room temperature in transport buffer (20 mM HEPES 7.3, 110 mM KOAC, 5 mM Na(OAc)$_2$, 2 mM Mg(OAc)$_2$, 2 mM dithiothreitol, 1 mM EGTA, 1 μg/ml leupeptin and pepstatin) (43). All of the proteins used in the nuclear import assay had a final purification step of gel filtration into transport buffer. Immediately after permeabilization, the cells were incubated with transport buffer containing 5 μM of RanQ69L and 150 μM of RanBP1 for 8 min at room temperature (47). RanQ69L induces importin β to dissociate from nucleoporins, thereby allowing it to be washed out of the cell (47, 48). The complex of RanQ69L and RanBP1 has also been shown to increase shuttling of export complexes (export factor + RanGTP) into the cytosol where they can be washed away (49). To begin the import process, the coverslips were inverted over a drop of transport buffer containing 3 μM importin β, 5 μM Ran, 150 nm NTF2, and 0.6 μM GST-GFP cargo as well as an energy regeneration system (0.1 mM ATP, 0.1 mM GTP, 10 mM creatine phosphate, and 20 units/ml creatine phosphokinase (44)). Import was allowed to go to equilibrium at 30 °C for 30 min. The cells were immediately fixed, washed with ice-cold phosphate-buffered saline, and then visualized

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Using a Zeiss Axioskope under a 40× air objective. To deplete energy, the cells were incubated with 10 mM glucose, 1600 μg/ml hexokinase, and 100 mM ADP for 20 min at room temperature before the import reaction (50). The images were quantified using Image J software. Fluorescence intensity at both the nuclear rim and the center were measured taking care not to include the nucleoli. Cytosolic extract from HeLa cells was prepared as previously described (51).

Halo Bead and Pulldown Assay—Nup358, Nup214, Nup62, and Nup153 fused to GST were immobilized on glutathione beads and resuspended in binding buffer (20 mM HEPES, pH 7.0, 150 mM KOAc, 2 mM Mg(OAc)₂, 1 mM dithiothreitol, and 0.1% Tween) to a 50% slurry. On a glass slide, 0.75 μL of GST-nups were mixed with 0.75 μL of either free mCh-importin β or mCh-importin β precomplexed to IBB domains; 0.5 μL of 4× EHBn buffer (40 mM EDTA, 2% 1–6 hexanediol, 40 mg/ml bovine serum albumin, and 500 mM NaCl) was mixed to the drop. The beads were imaged using a Leika CTR5000 with a 20× air objective. Pulldown assay was carried out as previously described (10).

Surface Plasmon Resonance (SPR)—SPR experiments were carried out on a Biacore 3000 instrument, equilibrated at 25 °C in HBS-EP buffer (0.01 M HEPES, pH 7.4, 0.15 M NaCl, 3 mM EDTA, 0.005% Surfactant P20; GE Healthcare Bio-Sciences AB, Uppsala, Sweden). We captured purified recombinant GST-Nup62 on a CM5 sensor chip coupled with immobilized goat anti-GST antibody. Samples of importin β, both alone and prebound to IBB domains, were applied to the chip at various concentrations in HBS-EP buffer. Response units were recorded at a flow rate of 30 μl min⁻¹ with injection time and wait time after injection of 3 and 10 min, respectively. Each experiment was repeated four times. The apparent association and dissociation rate constants and χ² values were calculated using 1:1 Langmuir model in BIAevaluation software version 4.1 (Bia-core AB). A comprehensive list of all kinetic parameters is in supplemental Table S1.

Electron Microscopy—Colloidal gold particles (9 nm in diameter) were prepared and conjugated with the importin β-IBB complexes as previously described (33, 52). Xenopus oocytes (stage VI) were microinjected into their cytoplasm with 50 nL of gold-conjugated complexes, and the injected oocytes were incubated at room temperature for 1 h and processed for embedding/thin sectioning electron microscopy as previously described (33, 52). Micrographs were digitally recorded with an H-700 transmission electron microscopy (Hitachi). For quantification the position of gold particles associated with NPCs were determined from micrographs of cross-sections along the nuclear envelope. Only NPCs that were clearly sectioned at their central plane (which is indicated by a continuity of the outer and inner nuclear membrane) were quantified. 100 NPCs from four different experiments/sample were analyzed. Gold particles were predominantly associated with the central channel of the NPC (defined as vertical distances of 10 ± 5 nm perpendicular to the central plane of the nuclear envelope in both the cytoplasmic and nuclear face of the NPC) and within the nuclear basket (defined as vertical distances of 60–100 nm perpendicular to the central plane of the nuclear envelope in the nuclear face of the NPC).

RESULTS

The Nature of the IBB Domain Affects the Energy Requirement for Nuclear Import—The karyophilic properties of the import adaptors importin α and SNF1 are mediated by the IBB domain. Structural superimposition of full-length importin β bound to sIBB(25–65) (10) and αIBB domain (7) (both complexes solved crystallographically to 2.3Å resolution) reveals that the sIBB domain is slightly shorter than its counterpart (35 versus 41 Å) because of a 3-amino acid gap between residues 46 and 47 (Fig. 1A). Despite this difference in length, the conformation of the importin β backbone is very similar in both complexes (root mean square deviation, ~0.965 Å), suggesting that the flexible nature of the HEAT-repeated architecture can compensate for the shorter sIBB helix (Fig. 1B). To determine how the length of the IBB domain affects the binding affinity for importin β, we filled the 3-amino acid gap in the sIBB helix by introducing three alanines between residues 46 and 47 (construct sIBB(25–3A-65)). This insertion is not expected to add binding determinants for importin β but simply extends the length of the sIBB C-terminal helix to match that seen in the αIBB domain (Fig. 1C). Using SPR, we measured the affinity of sIBB(25–3A-65) for importin β. We found that extending the sIBB helix increased the affinity for importin β as compared with the shorter sIBB(25–65) (Kd = ~10.6 ± 3.0 nM versus Kd = ~15.3 ± 5.8 nM) (10) and slowed down its dissociation (koff = 5.15 e⁴ ± 0.5 e⁴ s⁻¹ versus koff = 8.24 e⁴ ± 0.5 e⁴ s⁻¹) (10) (supplemental Fig. S1). We conclude that the length of the sIBB C-terminal helix and not just its amino acid sequence determines the binding affinity of the sIBB domain for importin β.

The IBB Domain Affects the Energy Requirement for Nuclear Import in Permeabilized Cells—To understand how the nature of the IBB domain affects the karyophilic properties of importin β, we compared the nuclear import of sIBB to the classical αIBB domain using a nuclear import assay in digitonin-permeabilized HeLa cells. We generated four nondiffusible import cargos consisting of a chimera of GST-GFP (molecular mass, 23 kDa) fused to importin β, lacking (stage VI) were microinjected into their cytoplasm with 50 nL of gold-conjugated complexes, and the injected oocytes were incubated at room temperature for 1 h and processed for embedding/thin sectioning electron microscopy as previously described (33, 52). Micrographs were digitally recorded with an H-700 transmission electron microscopy (Hitachi). For quantification the position of gold particles associated with NPCs were determined from micrographs of cross-sections along the nuclear envelope. Only NPCs that were clearly sectioned at their central plane (which is indicated by a continuity of the outer and inner nuclear membrane) were quantified. 100 NPCs from four different experiments/sample were analyzed. Gold particles were predominantly associated with the central channel of the NPC (defined as vertical distances of 10 ± 5 nm perpendicular to the central plane of the nuclear envelope in both the cytoplasmic and nuclear face of the NPC) and within the nuclear basket (defined as vertical distances of 60–100 nm perpendicular to the central plane of the nuclear envelope in the nuclear face of the NPC).
nuclear import with recombinant factors was less efficient for sIBB cargos as compared with the classical IBB substrate (Fig. 2B). Lack of exogenous Ran did not result in rim staining, as seen for the IBB cargo but gave a diffuse, faint intranuclear fluorescence (Fig. 2A, second through fourth rows, fourth column). This suggests that in the absence of Ran, sIBB cargos do not stick to the NPC but slowly accumulate into the cell nucleus over time. Although in our assay HeLa cells were extensively depleted of endogenous factors, it is possible that trace amounts of Ran left in the system may have accounted for the residual import observed in the absence of Ran. To corroborate this idea, if endogenous Ran was not depleted from HeLa cells by preincubation with RanQ69L and RanBP1, efficient accumulation of all cargos was observed in the absence of exogenous Ran (Fig. 2A, first through fourth rows, sixth column), as previously reported by Huber et al. (31). However, the absolute requirement for Ran was demonstrated by the fact that Δ44-importin β completely inhibited nuclear import of sIBB cargos.

**FIGURE 1. A three amino acid gap in the C-terminal helix of the sIBB domain.** A, sequence alignment of IBB domains used in this study. Identical residues are shown in red, and the triple alanine insertion in construct sIBB(25–3A-65) is underlined in blue. In green is the recently identified nuclear export signal (14, 15). The schematic topology of the IBB domain is illustrated on top of the sequence alignment. B, crystal structures of importin β bound to sIBB (left, Protein Data Bank code 2P8Q) (10) and αIBB (right, Protein Data Bank code 1QGK) (7). Importin β is shown in gray, with sIBB and αIBB in magenta and cyan, respectively. C, zoomed in view of sIBB(25–65) superimposed to the αIBB(11–54). The C-terminal helix in the sIBB domain is −5 Å shorter than in αIBB because of a 3-amino acid gap between residues 46 and 47.
FIGURE 2. Nuclear import of nondiffusible sIBB cargo in digitonin-permeabilized HeLa cells requires Ran. A, nuclear import of four different GST-GFP-sIBB cargos (row headings). The first through fifth columns include import reactions where HeLa were preincubated with RanBP1 and RanQ69L. The reactions were carried out in the presence of buffer (first column), cytosolic extracts (second column), all factors (importin \( \beta \), NTF2, Ran, plus energy) (third column), no Ran (importin \( \beta \), NTF2, plus energy) (fourth column), and \( \Delta 44\beta \) (\( \Delta 44\)-imp \( \beta \), NTF2, Ran, plus energy) (fifth column). The sixth column shows an import reaction with no Ran (importin \( \beta \), NTF2, plus energy) using HeLa cells that were not pretreated with RanQ69L/RanBP1 wash. Under these conditions, each cargo was imported in the absence of exogenous Ran. B, quantification of nuclear fluorescence at the center of the cell nucleus and at the nuclear rim is shown in white and black columns, respectively. The error bars represent the standard deviation of at least three independent nuclear import assays.

| A | Buffer | Cystosol | Factors | No Ran | \( \Delta 44\beta \) | No wash |
|---|--------|----------|---------|--------|-----------------|---------|
| sIBB |  |  |  |  |  |  |
| (1-65) |  |  |  |  |  |  |
| sIBB |  |  |  |  |  |  |
| (25-65) |  |  |  |  |  |  |
| sIBB |  |  |  |  |  |  |
| (25-3A-65) |  |  |  |  |  |  |

| B | αIBB | sIBB(1-65) | sIBB(25-65) | sIBB(25-3A-65) |
|---|------|-----------|-------------|-------------|

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as much as blocked αIBB import (Fig. 2A, first through fourth rows, fifth column). Therefore, the efficient import of IBB cargos observed in permeabilized cells not pretreated with RanBP1/RanQ69L (Fig. 2A, sixth column) was due to endogenous Ran remaining in the system. In contrast to a previous report by Huber et al. (31), our data unambiguously confirm that Ran is essential for nuclear import of SNPs.

In a second set of experiments, we probed the ability of αIBB(1–65) to be imported into the nucleus of permeabilized cells in the absence of exogenous nucleotide and an energy regenerating system (indicated in Fig. 3A as Energy). Interestingly, whereas αIBB import was nearly entirely abolished in the absence of energy (Fig. 3A), the lack of exogenous nucleotide reduced nuclear accumulation of sIBB(1–65) by only ~30% (Fig. 3B). These data indicate that both the αIBB and sIBB domains promote efficient nuclear import in permeabilized cells in the presence of importin β and Ran, although the sIBB has a much reduced energy requirement. Because nucleotide is used by Ran to displace the import complex from high affinity nup-binding sites as well as release the import cargo into the cell nucleus, we hypothesized that the reduced energy requirement for sIBB(1–65) nuclear import is caused by the reduced avidity of the importin β-sIBB import complex for FG-rich nups during translocation.

The IBB Domain Modulates the Affinity of Importin β for FG-rich Nups—To compare the affinity of importin β for FG nups in the presence or absence of IBB domains, we first assembled homogeneous complexes of importin β fully saturated by IBB domains. On a native agarose gel, all of the importin β/IBB complexes were significantly shifted as compared with free protein, suggesting that no free importin β was copurified with the complex (supplemental Fig. S2). In a first set of experiments we used the bead halo assay (53, 54) to probe the affinity of importin β fused to mCherry (mCh-importin β) for the FG-rich domains of nups Nup358, Nup214, Nup62, and Nup153 immobilized on glutathione beads (Fig. 4A). In this assay, high affinity interactions ($K_d = < 1 \mu M$) usually result in complete titration of the fluorescent ligand onto the bead, with consequent darkening of the background surrounding the beads and appearance of a fluorescent halo around the surface. In contrast, low affinity interactions give a less intense halo around beads as well as bright fluorescence in the surrounding solution because of the unbound fluorescent ligand left in solution (53, 54). Free mCh-importin β alone or prebound to αIBB and sIBB domains incubated with control glutathione beads coupled to GST resulted in no halo around the beads and high fluorescence background (Fig. 4A, first row). When Nup358 and Nup214 were tested, we observed formation of a fluorescent halo for the first and diffuse fluorescence around the entire bead for the latter (Fig. 4A, second and third rows, respectively). Nup214 beads were always entirely coated by fluorescent importin β, regardless of the presence of IBB domains, and high fluorescent background was observed in solution (Fig. 4A, third row). Diffuse fluorescence coating the entire bead, as opposed to a simple halo still denotes an interaction and may reflect the filamentous nature of Nup214 and its ability to coat the surface of the entire bead when bound to fluorescent importin β (53). When Nup26 was tested, we found that the affinity for importin β varied significantly with respect to the bound IBB. Whereas a bright halo and low background were observed for Nup62 beads incubated with mCh-importin β alone or precomplexed with a αIBB or sIBB(25–3A-65) domain (Fig. 4A, fourth row), a much higher background and a lighter halo was seen with mCh-importin β bound to sIBB(1–65) or sIBB(25–65) (Fig. 4A, fourth row). This was clearly indicative of decreased binding affinity of importin β for Nup62 in the presence of the sIBB domain. In contrast, GST-Nup153 gave strong halo fluorescence and very low background regardless of the presence of IBBs bound to importin β (Fig. 4A, fifth row).

The reduced affinity of importin β bound to sIBB domain for Nup62 was also confirmed by pulldown assay. Although this binding technique is also semi-quantitative, it does not require fusing importin β to the bulky mCherry fluorophore, which may affect the binding to FG nups. By pulldown assay, free importin β as well as importin β complexed to αIBB were efficiently pulled down by Nup62 (Fig. 4B, lanes 4 and 5). In contrast, importin β bound to sIBB(1–65) or sIBB(25–65) showed dramatically reduced binding to Nup62 (Fig. 4B, lanes 6 and 7). Strikingly, the addition of three alanines within the IBB C-terminal helix in the construct sIBB(25–3A-65) restored binding of importin β to Nup62 (Fig. 4B, lane 8). Thus, both the bead halo and pulldown assays provide initial evidence that the equilibrium binding affinity of importin β for certain FG nups is modulated by the nature of the IBB domain. The sIBB domain, and not the αIBB domain, reduces the affinity of importin β for Nup62, which forms the central channel of the NPC (55).

Importin β Bound to the sIBB Domain Has a ~200-fold Reduced Affinity for Nup62—We next used SPR to obtain a quantitative kinetic description of the reduced affinity of importin β for Nup62 in the presence of the sIBB domain. Increasing concentrations of free importin β or importin β precomplexed to IBB domains was flowed into the cell containing GST-Nup62 immobilized on a biosensor surface. Each interaction was measured using 10 different concentrations of ligand, and each concentration was repeated four times (Fig. 5 and supplemental Table S1). Interestingly, free importin β had the highest affinity for Nup62, with a $K_d$ value of ~8.04 ± 0.2 nM (Fig. 5A). This value is higher than the $K_d$ previously reported using a microtiter plate binding assay ($K_d = 100 \pm 8$ nM) (27), which may reflect differences in the binding technique or fitting models used to calculate affinities. In the presence of the αIBB domain, the affinity for Nup62 was reduced by ~40-fold ($K_d = 335 \pm 90$ nM; Fig. 5B) as compared with free importin β. Strikingly, in the presence of the sIBB(1–65) and sIBB(25–65), the affinity of importin β for Nup62 dropped into the micromolar range ($K_d = 1.58 \pm 0.4$ and ~0.86 ± 0.07 μM, respectively) (Fig. 5, C and D). In agreement with bead halo and pulldown assays, the affinity of importin β bound to the sIBB(25–3A-65) for Nup62 was comparable with that seen with the αIBB domain ($K_d = 255 \pm 30$ nM) (Fig. 5E). It should be pointed out that in a previous study (27), identical affinity of importin β for Nup62 in the presence or absence of αIBB domain was measured. This is certainly not true in our case, where the classical αIBB domain significantly reduced the affinity of importin β for Nup62 ($K_d = 335$ nM versus 8.04 nM). Notably, the accurate and reproducible $K_d$ val-
FIGURE 3. Reduced energy requirement for nuclear import of nondiffusable sIBB cargo. A, nuclear import assay in digitonin-permeabilized HeLa cells of GST-GFP-sIBB(1–65) and GST-GFP-sIBB cargos (row headings) in the presence of recombinant factors (importin β, NTF2, Ran) under no energy conditions (left column) or in the presence of energy (right column). In both reactions, HeLa cells were pretreated with RanQ69L/RanBP1 prior to carrying out the import reaction. B, quantification of intranuclear and nuclear rim fluorescence intensity (white and black columns, respectively) with the error bars representing the standard deviation of at least three independent assays.
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ues for Nup62 could be measured only when homogeneous importin β-IBB complexes that did not contain free importin β (supplemental Fig. S2) were used in the SPR assay. Thus, kinetic analysis by SPR demonstrates that the binding affinity of importin β for Nup62 is reduced by ~200-fold when the protein is bound to the sIBB domain. The length of the sIBB C-terminal helix is an important determinant in modulating the affinity of importin β for Nup62.

The IBB Domain Regulates the Persistence of Import Complex at the NPC—To investigate how the affinity of importin β-IBB complexes for Nup62 correlates with the persistence of the import complex inside the NPC, we carried out electron microscopy analysis of transport reactions containing colloidal gold (9 nm in diameter) coated with importin β-IBB complexes (Fig. 6). Samples of importin β-IBB-gold complexes were microinjected in *Xenopus* oocytes, and after appropriate fixation and embedding the oocytes were sectioned and visualized by electron microscopy. Fig. 6 (A–D) shows thin section electron micrographs of representative *in vivo* import reactions carried out in the presence of four distinct importin β-IBB-gold complexes. Gold particles were predominately associated with the cytoplasmic face of the NPC. Quantitative analysis of the gold particle position with respect to the central plane of the NPC revealed gold particles in the central channel and at the NPC nuclear basket. Quantification of the number of gold particles at these positions yielded the histograms shown in Fig. 6 (E and F, respectively). These data clearly demonstrate that although the classical εIBB cargo localizes at equilibrium to the cytoplasmic filaments, inside the NPC central channel and at the basket (Fig. 6, A, E, and F), import complexes containing either full-length sIBB(1–65) or the shorter sIBB(25–65) were rarely found at the center of the NPC (Fig. 6, B, D, and E), where Nup62 is highly concentrated. Interestingly, sIBB(25–3A-65) displayed similar persistence at the center of the NPC as observed for the εIBB cargo (Fig. 6, C and E), which is consistent with the higher affinity for Nup62. Furthermore, we noticed a remarkable difference in the persistence of sIBB(1–65) import complex at the nuclear basket, as compared with sIBB(25–65) and εIBB. Less than 35% of the sIBB(1–65) complexes were localized at the nuclear basket (Fig. 6, B and F), which is nearly half of the sIBB(25–65), sIBB(25–3A-65), and...
αIBB import complexes seen at the nuclear basket (Fig. 6, A–D and F). This agrees well with the previous observation made by Rollenhagen et al. (33). The fact that either truncating the first 24 residues of SNP1 (construct sIBB(25–65)) or extending the sIBB helix (construct sIBB(25–3A-65)) rescued high affinity binding to the nuclear basket, suggests different mechanisms for targeting of the import complex at the basket may play a role in this phenomenon. Although the sIBB(25–3A-65) is likely to

**FIGURE 5.** A 200-fold drop in the binding affinity of importin β for Nup62 in the presence of sIBB domain. SPR analysis of the interaction of importin β alone (A) or bound to αIBB (B), sIBB(1–65) (C), sIBB(25–65) (D), or sIBB(25–3A-65) (E) with Nup62 captured onto a sensor chip. In each sensogram, raw data are shown as open circles, and fit curves are shown as black lines for five different concentrations. A representative $K_d$ for each interaction is shown next to the relative sensogram. A complete list of kinetic parameters is in supplemental Table S1.
**FIGURE 6.** Decreased persistence of the sIBB import complex at the center of the nuclear pore complex in *Xenopus* oocytes. A–D, thin section electron micrographs of in vivo import reactions from *Xenopus* oocytes injected with gold-conjugated complexes of importin β bound to αIBB (A), sIBB(1–65) (B), sIBB(25–3A-65) (C), and sIBB(25–65) (D). E and F, quantification of gold particle positions expressed as percentages of NPCs with gold particles seen at the NPC central channel (E) and at the nuclear basket (F). In the presence of sIBB(1–65) and sIBB(25–65), the persistence of the import complex in NPC central channel is drastically reduced as compared with the import complex containing αIBB and sIBB(25–3A-65). c and n indicate cytoplasm and nucleus, respectively. Gold particles associated with 100 NPCs/condition from four different experiments were analyzed. Imp, importin.
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have higher persistence at the basket because of its αIBB-like nature, the shorter sIBB(25–65) lacks the N-terminal sn(1–24), which has high sequence identity to region (1011–1035) of Nup153 (10) and contains a functional nuclear export signal (14). In conclusion, the structure of the IBB domain regulates the persistence of the import complex in the NPC. In agreement with its lower avidity for Nup62, importin β bound to the sIBB(1–65) presents a dramatically reduced persistence inside the NPC gated channel and, to a lesser extent, the nuclear basket.

**DISCUSSION**

In this study, we provide evidence that the “nature” of the sIBB domain modulates the affinity of importin β for the NPC. We show that the length of the sIBB C-terminal helix affects both binding affinity for importin β and sIBB karyopherin properties. Because primary sequence dictates polypeptide main chain propensity to adopt helical folding, the sIBB amino acid sequence, which is distinct from αIBB, is also presumed to be an important determinant, intimately coupled with helix length.

The sIBB domain was previously reported to mediate Ran and energy-independent nuclear import of β-galactosidase, in permeabilized cells (31). In our study, we reinvestigated this import pathway but replaced the tetrameric import cargo sIBB-β-galactosidase (31, 38), which contains four sIBB domains, with a chimera of GST-GFP (molecular mass, ~55 kDa) fused to one sIBB domain. In contrast to previous data (31), we found that, in vitro, nuclear import of sIBB(1–65) is strictly dependent on Ran and has ~70% reduced requirement for exogenous energy as compared with the classical αIBB cargo. The need for Ran is confirmed by the dominant negative function of Δ44-importin β, which completely abolishes nuclear import of sIBB cargo. Furthermore, in our hands, reproducible and meaningful analysis of sIBB and αIBB nuclear import was possible only if HeLa cells were preincubated with RanBP1 and RanQ69L to deplete endogenous Ran and β-karyopherins. Without this pre-treatment, nuclear import of sIBB cargo proceeded efficiently in the apparent absence of exogenous Ran (Fig. 2A), as previously reported by Huber et al. (31). Although we found that nuclear import of sIBB cargo needs Ran as much as αIBB does, we determined a lower energy requirement for sIBB as compared with its counterpart. The addition of a nucleotide triphosphate (ATP or GTP) in the import reaction is thought to re-charge Ran, which clears the passage of import complexes through the NPC. The reduced energy requirement observed for sIBB(1–65) nuclear import led us to hypothesize that in complex with the sIBB, importin β has a reduced avidity for nups, thereby resulting in fewer moles of GTP hydrolyzed per passage through the NPC. This hypothesis was tested using equilibrium and kinetic binding assays. Interestingly, we found that the affinity of importin β for Nup62, which forms the central channel of the NPC (55), is specifically reduced in complex with the sIBB domain. SPR revealed a ~200-fold drop in the binding affinity of importin β for Nup62 in the presence of sIBB(1–65) ($K_d = 1.58 \, \mu M$ versus 8.04 nM) and a ~40-fold drop in the presence of the αIBB domain.

How can binding of an IBB domain affect the affinity of importin β for FG repeats? The IBB domain is approximately one-twentieth of the importin β mass. It is tightly bound to the inner surface of the protein, whereas FG-binding pockets are harbored on the outer surface of the protein. Two bipartite FG-binding sites have been experimentally uncovered in importin β between HEATs 5–7 and 14–16 (24, 26), and an additional six sites were demonstrated in silico (56). Bound to IBB domains, importin β adopts a highly strained conformation (10). In analogy to the molecular description of karyopherins introduced by Matsuura and Stewart (16) to describe the strained conformation of the export factor Cse1p bound to Kap60p and RanGTP, importin β bound to the IBB domain is comparable with a “spring-loaded” solenoid. Although the structure of importin β in complex with the sIBB and αIBB domain are crystallographically very similar (10), our data suggest that there exist significant differences in its affinity for Nup62. We propose that the strain of the spring-loaded protein is much greater when importin β is bound to the sIBB domain. The sIBB helix is in fact ~5 Å shorter than its counterpart αIBB (Fig. 1), which implies that a greater degree of contortion in importin β is required to generate the electrostatic complementarity that drives complex formation. If the primary consequence of a spring-loaded conformation is the ability to undergo spontaneous changes (16), we hypothesize that the highly strained conformation of importin β in complex with the sIBB increases the breathing motion of the outer protein surface, thus weakening the interaction with FG nups and leading to the spontaneous dissociation from FG sites. The hypothesis of a highly strained, metastable conformation of importin β bound to the sIBB helix may help reconcile the crystallographic observation of an open conformation of ΔN127-importin β bound to the sIBB(1–65), recently reported by Wohlwend et al. (57). This structure was in fact obtained under high ionic strength (~1 M ammonium sulfate) and shows no density for sIBB N-terminal residues 1–40, together with a fully open and unstrained conformation of importin β. As the ionic strength increases, the electrostatic interactions that stabilize sIBB binding to importin β weaken. This results in a loss of the enthalpic energy necessary to outweigh the entropic cost of being stained. As a result, ΔN127-importin β relaxes into an open conformation somewhat similar to the structure of Kap95p bound to RanGTP (13).

In agreement with our model, filling the 3-residue gap in the sIBB C-terminal helix with alanines (construct sIBB(25–3A-25)) resulted in at least three observable phenomena. First, it increased the binding affinity of the sIBB(25–3A-65) for importin β nearly 2-fold, likely by reducing the stretching of importin β around the sIBB helix. Second, bound to the sIBB(25–3A-65) importin β regained high affinity binding to Nup62, likely because of an increased avidity for FG repeats. Third, the importin β/sIBB(25–3A-65) complex was found to persist in the center of the NPC dramatically longer than the sIBB(25–65) complex. Thus, the nature of the sIBB C-terminal helix plays a critical role in determining the behavior of importin β inside the NPC. However, the sIBB(1–65) was found to lower the affinity of importin β for only one of the five FG nups tested in our study, Nup62, that forms the central channel of the NPC (55). This suggests that specific differences must exist within...
different nups, based on the number, density, and distribution of FG motifs within their sequence.

In conclusion, we propose that the reduced persistence of importin β bound to the s1BB(1–65) in the NPC reflects a mechanism to avoid clogging of the NPC by the importin β-SNP1-snRNPs import complex. Considering the large size of the RNA splicing machinery, essential to RNA metabolism.

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