β-Catenin Shows an Overlapping Sequence Requirement but Distinct Molecular Interactions for Its Bidirectional Passage through Nuclear Pores

Makiko Koike‡, Shingo Kose‡, Maiko Furuta‡‡, Naoko Taniguchi‡, Fumihiko Yokoya§, Yoshihiro Yoneda‡, and Naoko Imamoto††

From the §Cellular Dynamics Laboratory, Discovery Research Institute, RIKEN, 2-1 Hirosawa, Wako, Saitama 351-0198 and the $Department of Frontier Biosciences, Graduate School of Frontier Biosciences, Osaka University, 2-2 Yamada-oka, Suita, Osaka 565-0871, Japan

β-Catenin is an example of a typical molecule that can be translocated bidirectionally through nuclear pore complexes (NPCs) on its own in a facilitated manner. In this work the nuclear import and export of β-catenin were examined to compare the sequence requirement of this molecule and to determine whether molecular interactions required for its bidirectional NPC passage are distinct or not. Deletion analysis of β-catenin revealed that armadillo repeats 10–12 and the C terminus comprise the minimum region necessary for nuclear migration activity. Further dissection of this fragment showed that the C terminus tail plays an essential role in nuclear migration. The region of β-catenin required for export substantially overlapped the region required for import. Therefore, the NPC translocation of β-catenin is apparently reversible, which is consistent with findings reported previously. However, different translocating molecules blocked nuclear import and export of β-catenin differentially. The data herein indicate that β-catenin shows an overlapping sequence requirement for its import and export but that bidirectional movement through the NPC proceeds through distinct molecular interactions.

The bidirectional exchange of macromolecules is mediated by the nuclear pore complex (NPC) embedded in lipid bilayers of the nuclear envelope. NPC is one of the largest macromolecular assemblies that is currently known in eukaryotic cells, with an estimated mass of about 66 MDa, consisting of multiple copies of about 30 different proteins. Molecules smaller than 40–60 kDa (or 9 nm in diameter) can diffuse through NPC, whereas bidirectional transport of larger molecules across the NPC occurs by active or facilitated mechanisms (for review, see Refs. 1–4). Many types of transport receptors mediate active nucleoplasmic trafficking. The best characterized class of nuclear transport receptors is a large family of importin-β-related proteins called importins or exportins (for review, see Refs. 5–9). Importins and exportins mediate the nuclear import and export of proteins as well as some RNAs. Small GTPase Ran, in its GTP form, which disassembles import complexes and assembles export complexes, is essential in these transport pathways (for review, see Refs. 5 and 6). The GTPase cycle of Ran, which generates RanGTP in the nucleus by the Ran guanine nucleotide exchange factor RCC1 (RanGEF) (10), and RanGDP in the cytoplasm by the Ran GTPase-activating protein (RanGAP) (11), creates a steep RanGTP gradient across the nuclear envelope (12) and assures the directional transport of importin/export cargoes (13). In addition to these receptors, the NXF family functions as a nuclear export receptor of mRNA (14–16), and p10/NTF2 mediates the nuclear import of RanGDP to replenish nuclear Ran (17). However, not all nucleocytoplasmic trafficking is mediated by receptor proteins. For example, β-catenin has been shown to migrate into the nucleus on its own (18).

β-Catenin is a structural component of adheren junctions, where it binds to the cytoplasmic domain of cadherin (19). It is also a transcriptional coactivator in Wnt signaling transduction. The nucleocytoplasmic concentration of β-catenin thus affect a variety of biological phenomena, including cell differentiation, cell proliferation, cell-cell interactions, cell-matrix interactions, and tumorigenesis (for review, see Refs. 20 and 21). Although nuclear transport in vivo is an energy-consuming process, a number of in vitro studies have demonstrated that the NPC translocation step of transport does not require any metabolic energy (22–26). In the importin/exportin pathways, metabolic energy is supplied by the Ran GTPase cycle (27), which operates on both sides of the NPC, to assure the directionality of cargo transport. In contrast, the NPC translocation process itself is Ran-independent and is generally considered to be nonvectorial. However, a direct comparative study of nuclear import and export has not yet been reported, and the issue of whether bidirectional passage through the NPC actually occurs via the same molecular interactions remains elusive.

The nuclear import of β-catenin does not require an energy-consuming process (18). On the other hand, different groups have proposed different mechanisms for the nuclear export of β-catenin. One is an involvement of exportin1 along with adenomatous polyposis coli (APC) protein (28, 29), and the other is the Ran-independent receptor-free export (30). Because β-catenin binds to many different proteins in both the cytoplasm and the nucleus, and its binding partners differ in different cells or

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To whom correspondence should be addressed. E-mail: namamoto@riken.jp.

The abbreviations used are: NPC, nuclear pore complex; ARM (R) repeat, armadillo repeat; BSA, bovine serum albumin; CAS, cellular apoptosis susceptibility; GFP, green fluorescent protein; GST, glutathione S-transferase; WGA, wheat germ agglutinin.

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under different cellular conditions, different conclusions have been drawn, depending on the cellular system being studied.

Studies using a digitonin-permeabilized cell-free import and export assay, in conjunction with cytoplasmic and nuclear injection, allowed us to examine the nucleocytoplasmic shuttling activities of β-catenin directly. In this work, the sequence of β-catenin required for its import and export was examined. We found that armadillo (ARM) repeats 10–12 and the C terminus tail of β-catenin constitute the minimum region necessary for both import and export, which occurs in a receptor-free and energy-independent manner. Further dissection of this fragment showed that the C terminus tail of β-catenin plays an essential role in both import and export. In agreement with previous indications that the NPC translocation step of transport is reversible, the sequences required for β-catenin import and export were found to overlap substantially. On the other hand, quantitative import and export analysis showed that the deletion mutant whose import rate was significantly slower than that of full-length β-catenin, efficiently exits the nucleus with a rate that was comparable with full-length β-catenin. Moreover, somewhat unexpectedly, different translocating molecules blocked the import and export of β-catenin differentially. Our present data indicate that a molecule that contains an overlapping sequence requirement for both its import and export can transit through the NPC into and out of nucleus using distinct molecular interactions.

**EXPERIMENTAL PROCEDURES**

**Cell Culture and Cell Fusion**

HeLa cells were incubated in Dulbecco’s modified Eagle’s medium supplemented with 5% fetal bovine serum at 37 °C and plated on glass bottom microwell dishes (MatTek Corp.) for microinjection experiments or on 8-well multistest slides (ICN Biomedicals) for in vitro transport assay.

For cell fusion, a few drops of 50% (w/v) polyethylene glycol (PEG 1500, Roche Applied Science) were added to cells that had been pre-washed with serum-free medium and then incubated for 2 min at room temperature. Homoyakarons were prepared 2–4 h prior to nuclear injection experiments.

**Expression and Purification of Recombinant Proteins**

Recombinant β-catenin (18), CAS (31), importin α (22), GTPase Ran (18), and importin β (22) were prepared as described previously. β-Catenin (N terminus and ARM repeats 1–9 (N-R9); amino acids 1–519), ARM repeats 10–12 (R10-12; amino acids 520–664), ARM repeats 10–12 and C terminus (R10-C; amino acids 520–781), ARM repeats 11–12 and C terminus (R11-C; amino acids 583–781), and C terminus (C; amino acids 665–781), were amplified by PCR using appropriate oligonucleotides (N-R9, 5′-GATCCGATCCATGGCTACTC-AAGCTGACC-3′ and 5′-CTACCGGGAAGGGCAAGTTCTGCAAT-CAAATCC-3′; R10-12, 5′-CCTACGGATCCTGCCCAGCAAATCATGCG-3′; R11-C, 5′-GATCCGATCCATGGCTACTC-AAGCTGACC-3′ and 5′-CTACGGATCCTGCCCAGCAAATCATGCG-3′; R10-C, 5′-CCTACGGATCCTGCCCAGCAAATCATGCG-3′ and 5′-CTACCCGGGCGGCGAGGCATGTATACAAACGGC-3′; R11-C, 5′-CTACCCGGGCGGCGAGGCATGTATACAAACGGC-3′ and 5′-CTACCCGGGCGGCGAGGCATGTATACAAACGGC-3′) and the bound proteins were eluted with 20 mM glutathione dissolved in buffer B (20 mM phosphate, pH 7.2, 20 mM NaCl, 2 mM dithiothreitol, 1 μg/ml each aprotinin, leupeptin, and pepstatin A). GST-FLAG-N-R9-His6 was purified further using nickel-nitritrotetraic acid-agarose according to the manufacturer’s recommendations. All recombinant proteins were purified on a Mono Q column with a linear gradient of buffer B containing 0.02–1.0 M NaCl. The peak fraction containing each recombinant protein was collected, desalted by passing through a PD10 column (Amersham Biosciences) equilibrated with buffer B, and concentrated by ultrafiltration on a Microcon 50 (Amicon).

**In Vitro Transport Assays**

**Import Assay**—Digitonin-permeabilized HeLa cells were prepared as described previously (18). Translocation was performed in 10 μl of testing solution containing the recombinant proteins dissolved in transport buffer (20 mM HEPES, pH 7.3, 110 mM potassium acetate, 2 mM magnesium acetate, 5 mM sodium acetate, 0.5 mM EGTA, 2 mM dithiothreitol, 1 μg/ml aprotinin, leupeptin, and pepstatin A, and 2% BSA). The composition and concentration of the recombinant proteins are described in each figure legend. Where indicated, an energy-regenerating system (1 mM ATP (Sigma), 5 mM phosphocreatine (Sigma), 20 units of creatine kinase (Sigma)) was included in the above 10-μl testing solution.

**Export Assay**—β-catenin and its deletion mutants were first subjected to the import reaction described above (first incubation). The cells were immediately washed twice with ice-cold transport buffer and then incubated in the 10 μl of testing solution containing wheat germ agglutinin (WGA) (EY Laboratories, Santa Matoe, CA), recombinant proteins, or an energy-regenerating system to examine export (second incubation). In all experiments, cells were washed and fixed with 3.7% formaldehyde after the incubation time, and then they were examined by fluorescence microscopy (Olympus IX70).

**Microinjection**

Recombinant β-catenin or its deletion mutants were injected through a glass capillary into the cytoplasm or nucleus as described previously (18). Where indicated, WGA or Cy3-labeled BSA (injection marker) was co-injected. After incubation for 30 min at 37 °C, the cells were fixed with 3.7% formaldehyde and examined by fluorescence microscopy.

**Quantification of Nuclear Import and Export**

The GFP intensity of the nucleus (excluding the signal at NPCs) at each time point was measured using MetaMorph (Universal Imaging Corp.). The background fluorescent intensity measured in the neighboring region where no cells was present was subtracted. The fitting curves were obtained by using the nonlinear regression program from Graph Pad Prism 4.0 (Graph Pad Software, Inc.).

To estimate the absolute nuclear concentration from GFP intensity, the nuclear adenine using MetaMorph (Universal Imaging Corp.). The background fluorescent intensity measured in the neighboring region where no cells was present was subtracted. The fitting curves were obtained by using the nonlinear regression program from Graph Pad Prism 4.0 (Graph Pad Software, Inc.).

**Other Methods**

To examine the localization of FLAG-N-R9-His6, the fixed cells were permeabilized with 0.5% Triton X-100 in phosphate-buffered saline for 5 min at room temperature, incubated with 3% skim milk in phosphate-buffered saline for 20 min, and then incubated with a mouse monoclonal antibody to the FLAG epitope (2 μg/ml, Sigma, FLAG M2 monoclonal antibody, F3165) or β-catenin (1 μg/ml, NanoTools Antikörperfachtechnik, β-CAT-TD1). The mouse antibodies were detected with Cy5-labeled goat antimouse antibody (Amersham Biosciences) and pEGX-2T vector containing the green fluorescent protein (GFP) (32). Recombinant proteins were expressed by induction with 0.2 μM isopropyl-1-thio-β-D-galactopyranoside for 12 h at 18 °C, either in Escherichia coli strain BL21(DE3) (Gst-N-R9, Gst-R10–12, Gst-R10-C, Gst-R11-C, Gst-C, Gst-R10-C-GFP and Gst-C-GFP) or in E. coli strain DH5α (Gst-R10–12–GFP, Gst-R11-C-GFP, and Gst-FLAG-N-R9-His6). After expression, the bacteria were lysed in buffer A (50 mM Tris-HCl, pH 8.0, 50 mM NaCl, 2 mM dithiothreitol, 1 μg/ml each aprotinin, leupeptin, and pepstatin A) by freeze-thaw and sonication and then clarified by centrifugation (45,000 rpm, 30 min). The resulting supernatant was incubated with glutathione-Sepharose 4B for 2 h at 4 °C, and the bound proteins were eluted with 20 μl of glutathione in buffer B (20 μM phosphate, pH 7.2, 20 mM NaCl, 2 mM dithiothreitol, 1 μg/ml each aprotinin, leupeptin, and pepstatin A). GST-FLAG-N-R9-His6 was purified further using nickel-nitritrotetraic acid-agarose according to the manufacturer’s recommendations. All recombinant proteins were purified on a Mono Q column with a linear gradient of buffer B containing 0.02–1.0 M NaCl. The peak fraction containing each recombinant protein was collected, desalted by passing through a PD10 column (Amersham Biosciences) equilibrated with buffer B, and concentrated by ultrafiltration on a Microcon 50 (Amicon).

**RESULTS**

**ARM Repeats 10–12 and the C Terminus of β-Catenin Are Required for Nuclear Migration—β-Catenin Has the Ability to undergo translocation through NPCs in a Ran-independent manner without the need for soluble factors or energy sources (18) such as cargo-free importin β. Based on its crystal structure, β-catenin can be subdivided into three major domains: the N
terminus (amino acids 1–140, indicated as N in this work), 12 ARM repeats (amino acids 141–664, ARM is indicated as R in this work), and the C terminus (amino acids 665–781, indicated as C in this work) (33). A comparison of the crystal structure of cargo-free importin β and β-catenin revealed a remarkable conformational resemblance between the HEAT repeats 4, 5, 6, 7, and 8 of importin β and the ARM repeats 9, 10, 11, and 12 of β-catenin (34). The HEAT repeats 4, 5, 6, 7, and 8 of importin β comprise the region that is necessary and sufficient for its NPC translocation (22, 35). This evidence prompted us to focus on the latter one-third of the β-catenin for its nuclear migrating activity.

To determine the region of β-catenin necessary and sufficient for its NPC passage, a variety of β-catenin deletions (Fig. 1) were expressed, purified to homogeneity, and tested as transport substrates. These fragments were tagged with GFP, a FLAG epitope, or GST and GFP in the case of small fragments below the diffusion size. To determine whether the region comprising the ARM repeats 10–12 played an important role in nuclear migration, we first examined the nuclear import activity of two fragments, one of which contained ARM repeats 10–12 and the C terminus (GST-R10-C-GFP), and other, the fragment containing the N terminus and ARM repeats 1–10 (GST-N-R9-His6). As shown in Fig. 2A, GST-R10-C-GFP migrated efficiently into the nucleus when injected into the cell cytoplasm, similar to full-length GFP-β-catenin. On the other hand, cytoplasmically injected FLAG-N-R9-His6 did not migrate into the nucleus (Fig. 2A). When examined in digitonin-permeabilized cells, GST-R10-C-GFP migrated rapidly into the nucleus in the absence of soluble factors or energy sources analogous to full-length GFP-β-catenin (Fig. 2A). In a similar experiment, FLAG-N-R9-His6 did not accumulate in the nucleus of digitonin-permeabilized cells (Fig. 2A). The nuclear migration of GST-R10-C-GFP was inhibited by WGA in digitonin-permeabilized cells, GST-R10-C-GFP migrated into the nucleus in both living cells and digitonin-permeabilized cells (Fig. 2A, c, d, m, and n). The nuclear migration of these fragments was inhibited by WGA (Fig. 2A, i, j, s, and t) and occurred in the absence of soluble factors and energy sources, indicating that these two fragments are able to migrate into the nucleus in a facilitated manner analogous to full-length β-catenin. However, we noticed that the relative import level of a fragment containing only the C terminus (GST-C-GFP) or ARM repeats 11, 12, and the C terminus (GST-R11-C-GFP) was weaker than that for full-length β-catenin or a fragment containing ARM repeats 10–12 and C terminus (GST-R10-C-GFP; compare Fig. 2A, n and m, with h and l).

In the previous study, we showed that β-catenin does not accumulate into the nucleus of digitonin-permeabilized Madin-Darby bovine kidney cells against the concentration gradients (18), and this was similarly observed with a permeabilized HeLa cells (see supplemental data, Fig. 2S). Quantification of import showed that β-catenin fragments containing ARM repeats 10–12 and C terminus (GST-R10-C-GFP) and ARM repeats 11, 12, and the C terminus (GST-R11-C-GFP) also equilibrate between the nucleus and the cytoplasm like full-length β-catenin (Fig. 2B). However, GST-R11-C-GFP took much longer time to reach the equilibrium (half-maximum 18.6 min) compared with full-length β-catenin (half-maximum 1.7 min), and a slight delay was observed with GST-R10-C-GFP (half-maximum 6.4 min). In the case of the β-catenin fragment containing only the C terminus (GST-C-GFP), it did not reach equilibrium throughout the 40-min incubation time.

We next examined the issue of whether full-length β-catenin and β-catenin deletions involving the C terminus could mutually compete with each other for nuclear migration. As shown in Fig. 3, A and B, the addition of nontagged full-length β-catenin inhibited the nuclear import of GST-R10-C-GFP, GST-R11-C-GFP, and GST-C-GFP. Inverse experiments show that the addition of R10-C, R11-C, or C inhibited the nuclear import of full-length GFP-β-catenin (Fig. 3, C and D). The addition of a fragment lacking the C terminus (GST-R10–12), which did not migrate into the nucleus itself, had no effect on the nuclear import of GFP-β-catenin. A fragment containing R10-C inhibited the nuclear import of GFP-β-catenin almost as efficiently as full-length β-catenin (Fig. 3, C and D, see also supplemental data, Fig. 3S), but the inhibitory effect of R11-C and C was clearly weaker than that observed for R10-C. Although a delay was observed with R10-C to reach the equilibrium (Fig. 2B), the competition results show that R10-C interacts quite efficiently with the sites where full-length β-catenin interacts for nuclear migration.

From these results, we conclude that the C terminus tail of β-catenin plays an essential role in nuclear import but that the C terminus tail alone is not sufficient for complete nuclear import activity. Therefore, regions involving both ARM repeats
Fig. 2. Analysis of the sequence requirement for β-catenin import. A, 1 mg/ml recombinant full-length GFP-β-catenin (a and g), GST-R10-C-GFP (b and h), GST-R11-C-GFP (c and i), GST-C-GFP (d and j), FLAG-N-R9-His6 (e), or GST-R10–12-GFP (f) was injected into the cytoplasm of HeLa cells with (g–j) or without (a–f) 1 mg/ml WGA. Digitonin-permeabilized HeLa cells were incubated with 1 μM GFP-β-catenin (k and q), GST-R10-C-GFP (l and r), GST-R11-C-GFP (m and s), GST-C-GFP (n and t), FLAG-N-R9-His6 (o), or GST-R10–12-GFP (p) with (q–t) or without (k–p) WGA. After an import assay, GFP fusions were examined directly after fixation. FLAG-N-R9-His6 was detected by indirect immunofluorescence with an anti-FLAG mouse mAb (e) or anti-β-catenin β-CAT-7D11 mouse mAb (o), and Cy3-labeled anti-mouse goat IgG. B, quantification of nuclear import. Digitonin-permeabilized HeLa cells were incubated with 1 μM GFP-β-catenin and deletion mutants for various periods indicated in the figure at 30 °C. After fixation, the GFP intensity of the nucleus at each time point was measured. The fluorescent intensity was converted to protein concentration and plotted as described under “Experimental Procedures.” About 40 cells were analyzed for each time point.
R10–12 and the C terminus tail are required to retain nuclear migrating activity of β-catenin.

Sequence Determination of Region Required for β-Catenin Export—The energy-independent NPC translocation process has been proposed to be nonvectorial. However, whether the bidirectional NPC passage actually involves the same molecular interactions or not has not being clearly demonstrated. To obtain information on this, we determined whether β-catenin deletions that enter the nucleus were also capable of exiting the nucleus in the same manner.

To examine the export in living cells, HeLa cells were fused by treatment with polyethylene glycol, and recombinant β-catenin deletions were coinjected with an injection marker (BSA–Cy3) into one of the nuclei of a homokaryon. As shown in Fig. 4ad, β-catenin fragment R10-C, which possesses import activity, exited the nucleus and then entered all of the nuclei in homokaryons within a 30-min incubation in a manner identical to full-length β-catenin. β-Catenin fragment R10–12, which did not possess import activity, remained in the nucleus and did not exit (Fig. 4Ag). A β-catenin fragment comprising only the C terminus, which showed a weak nuclear import activity, was capable of exiting the nucleus (Fig. 4Am). However, in similar experiments, β-catenin fragment R11-C, which possesses the ability to migrate into the nucleus, did not exit the nucleus in living cells (Fig. 4Aj).

We next examined the export of β-catenin using digitonin-permeabilized HeLa cells incubated with 1 μM GST-R10-C-GFP (a and d), GST-R11-C-GFP (b and e), or GST-C-GFP (c and f) in the absence (upper panels) or presence (lower panels) of 5 μM nontagged full-length β-catenin. B, GFP intensity of each nucleus in A was measured as in Fig. 2B, and the mean intensity of 40 nuclei was plotted. The mean intensity of nuclei incubated with respective GFP-tagged mutant proteins in the absence of nontagged full-length β-catenin was taken as 100%. C, digitonin-permeabilized HeLa cells were incubated with 1 μM GFP-β-catenin in the absence (a) or presence of 10 μM competitor proteins; nontagged full-length β-catenin (b), GST-R10–12 (c), GST-R10-C (d), GST-R11-C (e), GST-C (f), or GST (g). D, nuclear GFP intensity of GFP-β-catenin in C was measured, and the mean intensity of 40 nuclei was plotted as in B. The nuclear GFP intensity of cells incubated with GFP-β-catenin in the absence of competitor molecules was taken as 100%.
length β-catenin and GST-10-C-GFP (discussed below). The inability of GST-R11-C-GFP to exit the nucleus indicates that its import rate is significantly slower than the import rate observed in Fig. 2B.

Our results show that β-catenin fragment R10-C, which was exclusively necessary for its nuclear import activity, possesses strong nuclear export activity as well. The β-catenin fragment comprising the C terminus, which plays an essential role in its nuclear import, also appears to possess an essential role in its nuclear export. However, β-catenin fragment R11-C, which possesses nuclear import activity, failed to exit the nucleus.

**Competition Studies Show That the Molecular Interactions Required for β-Catenin Import and Export Are Not Identical**—If a NPC translocation process is nonvectorial, molecules that migrate into the nucleus via direct interaction with some nuclear structure or component(s) inside the nucleus. Most of our results described above support this, i.e. that the regions required for β-catenin import and export substantially overlap, except for R11-C. The behavior of R11-C can be explained if this fragment is retained in the nucleus through binding to some nuclear structure or component(s) inside the nucleus. On the other hand, quantitative import and export analysis showed that deletion mutants, for example GST-C-GFP, whose import rate was significantly slower than that of full-length β-catenin, exit the nucleus efficiently. This implies that import and export reactions may not be exactly equivalent.

To determine whether the import and export of β-catenin actually occur through the same molecular interactions, we examined the ability of different molecules to inhibit the import and export of β-catenin by means of competition studies.

In initial experiments, we were surprised to find that β-catenin is unable to inhibit its own nuclear export at concentrations up to 10 μM, the maximum concentration possible to add in our assay (Fig. 5d). In contrast, the import of GFP-β-catenin was inhibited by 3 μM nontagged β-catenin, in both the first (Fig. 5b) and second (data not shown) incubations. Because β-catenin does not accumulate in the nucleus of HeLa cells against a concentration gradient, as we reported previously (18, supplemental data, Fig. 2S), the intra- and extranuclear concentrations of β-catenin would be the same during our import and export competition assays. Therefore, the same amount of competitor molecules would show the same effect on either the import or export of β-catenin if the molecular interactions required for both processes were identical (discussed below). The inability of β-catenin to inhibit its own export indicates that the binding sites necessary for β-catenin export did not reach saturation levels in our assay, up to 10 μM. On the other hand, the addition of 3 μM β-catenin saturated the binding sites required for its import.

We next examined the ability of different molecules, which are able to translocate through the NPC on their own, in terms of their ability to inhibit the export and import of β-catenin. The competitor molecules used in these experiments did not interact with β-catenin irrespective of the presence or absence of Ran (data not shown). Importin α inhibited both the nuclear import and export of β-catenin concentrations below 1 μM (Fig. 6, Ab, Af, and B). CAS, a member of the importin β family which functions as an export receptor for importin α (36), inhibited the export of β-catenin at a concentration of 10 μM, whereas 20 μM CAS, the maximum concentration possible to add, inhibited β-catenin import only slightly (Fig. 6, Ac, Ag, and C). Importin α, an ARM repeat containing protein that was recently shown to migrate into the nucleus on its own (37), had
no effect on either \( \beta \)-catenin export or import (Fig. 6, Ad, Ah, and D).

Finally, we examined the effect of importin \( \beta \) on the import and export of \( \beta \)-catenin in the presence of small GTPase Ran and ATP, a condition that generates RanGTP in the nucleus. The presence of ATP stimulated the export of \( \beta \)-catenin, leading to an apparent decrease in nuclear accumulation; however, this effect of ATP is Ran-independent (data not shown). Although importin \( \beta \) strongly inhibited both the import and export of \( \beta \)-catenin in the absence of Ran and ATP, importin \( \beta \) inhibited only the export of \( \beta \)-catenin in the presence of Ran and ATP (Fig. 7). These results also provide additional evidence that the molecular interactions required for \( \beta \)-catenin import and export through the NPC are not identical, as discussed below.

**DISCUSSION**

We and another group reported previously that \( \beta \)-catenin is able to shuttle between the cytoplasm and the nucleus without the need for soluble factors or energy sources (18, 30). \( \beta \)-Catenin was shown to bind directly to the phenylalanine-glycine (FG) repeat-containing NPC components (FG-nucleoporins) (38). Therefore it is likely that this molecule is translocated bidirectionally through the NPC via direct interactions with NPC components. In this work, the sequence requirement for import and export of \( \beta \)-catenin was analyzed, and the findings indicate that ARM repeats 10–12 and the C terminus tail of \( \beta \)-catenin comprise a minimum region necessary for both the import and export that occur in a receptor-free manner (Figs. 2 and 4). Deletion of the C terminus tail from this fragment abolished nuclear import and export activity, whereas the C terminus tail alone retained a weak nuclear import activity and sufficient export activity. These results show that the C terminus tail of \( \beta \)-catenin plays an important role both in nuclear import and export.

It has been proposed that the C terminus of \( \beta \)-catenin plays the role of a transcriptional activation domain (39, 40). The portion involving R10-C of \( \beta \)-catenin is also known to bind several factors that are important for \( \beta \)-catenin function. These factors include cAMP-response element-binding protein (CREB)-binding protein (CBP) and the closely related homolog p300 (41, 42), ICAT (Inhibition of \( \beta \)-catenin and Tcf) (43), Teashirt (Tsh) (44), and Chibby (45). CREB/p300, ICAT, Tsh, and Chibby bind to R11-C, R10–12, the C terminus, and R10-C of \( \beta \)-catenin, respectively. We propose a new role, in which the C terminus portion functions in the nucleocytoplasmic shuttling of \( \beta \)-catenin, and this region may interact with NPC components.

The NPC translocation process itself does not require any metabolic energy (22–26, 18), and the importance of specific interactions between various transport receptors with FG-nucleoporins has been well characterized (46, 47; for review, see Refs. 1 and 4). These findings led to the proposal that the NPC operates as a channel that selectively permits the facilitated transport of cargoes, when they are bound to transport receptors, or molecules that show the ability to bind FG-nucleoporins. The movement of molecules through the NPC has been...
HeLa cells were incubated with 0.5 μM importin β, CAS, and importin α on the import and export of GFP-β-catenin were examined. A, in import assay (a–d), digitonin-permeabilized HeLa cells were incubated with 0.5 μM GFP-β-catenin in the absence (a) or presence of 1 μM importin β (b), 10 μM CAS (c), or 10 μM importin α (d). In the export assay (e–h), after the import reaction with 0.5 μM GFP-β-catenin, the cells were reincubated with transport buffer in the absence (e) or presence of 1 μM importin β (f), 10 μM CAS (g), or 10 μM importin α (h). B–D, GFP intensity of each nucleus, incubated in the presence of increasing amounts of competitor molecules, was measured after the import and export assay, and mean fluorescent intensity was plotted as in Fig. 5. The competitor was importin β (B), CAS (C), and importin α (D). Error bars show the results of four independent experiments.

Debated extensively, and a variety of models based on either unidirectional movement or random diffusion have been proposed (48–50). However, although NPC translocation has been examined extensively with respect to nuclear import, nuclear export has not been. A direct comparison of nuclear import and export is crucial to our understanding of the movement of molecules through the NPC.

β-Catenin is a simple representative example for examining the bidirectional translocation through the NPC because this molecule transits through NPC on its own, and the small GTPase Ran affects neither its import nor export. We took advantage of the nature of β-catenin to examine import and export, our main aims being to compare the sequence requirement of this molecule and to obtain information as to whether molecular interactions required for bidirectional NPC passage are distinct or not. Our present data show that the region of β-catenin required for its import also overlaps with the region required for its export and that NPC translocation is apparently reversible. However, β-catenin deletion containing only the C terminus accumulated slowly into the nucleus (Fig. 2B) but exited efficiently (Fig. 4C). One explanation for these phenomena could be that import and export reactions are not exactly equivalent. Competition studies further support that the molecular interactions required for NPC passage of β-catenin into and out of the nucleus are not identical.

We reasoned that the inhibitory effect of competitor molecules against β-catenin import and export would be same if the molecular interactions required for bidirectional passage through NPC are identical, based on the following grounds. 1) β-Catenin does not accumulate in the nucleus against a concentration gradient (18 and supplemental data, Fig. 2S). Therefore, the extra- and intranuclear concentrations of β-catenin would be same during import and export competition studies. 2) Competitor molecules, such as importin β and CAS, would possess saturable NPC binding sites with a certain NPC binding affinity. Based on their binding affinity, competitor molecules would occupy a fixed number of NPC binding sites at each concentration incubated. 3) NPC binding sites for competitor molecules are not restricted to the cytoplasmic face of the NPC, even if competitor molecules were to be added from the cytoplasmic side because all molecules used in the present experiments show nucleocytoplasmic shuttling activity.

The results of our competition studies argue that the movement of β-catenin through the NPC into and out of the nucleus can be achieved through different affinities for NPC components. β-Catenin import was inhibited competitively by 3 μM β-catenin, whereas its export was not inhibited at concentrations of up to 10 μM (Fig. 5). β-Catenin export requires a specific region at the protein, which was inhibited by WGA (Fig. 4B), importin β, and CAS (Fig. 6). Therefore, β-catenin export requires specific molecular interactions at the NPC and is not a simple diffusion. The inability of β-cate-
and saturate the NPC binding sites necessary for its export. The slight inhibition of this amount of CAS saturated most NPC sites required for import and export of portin and ATP. A nin in the presence of GTPase Ran export of CAS were to be added. On the other hand, CAS inhibited the amount of CAS up to 20 NPC is much stronger than that of CAS. Because increasing the same NPC binding sites required for nuclear import of CAS were to be added. In the export assay (e–h), after the import reaction with 1 μM GFP-β-catenin, the cells were reincubated with transport buffer in the absence (e and f) or presence (g and h) of 3 μM importin β. In f and h, 4 μM GTPase Ran and ATP-regenerating systems were added in the second incubation. B, graphs: GFP intensity of each nucleus in A was measured, and the mean fluorescence of 40 nuclei was plotted. Nuclear GFP intensity of cells incubated GFP-β-catenin in the absence of competitor was taken as 100% for the import assay, and the second incubation performed in the presence of WGA was taken as 100% for the export assay.

In summary, our results indicate that the facilitated import and export of β-catenin proceed through distinct molecular interactions at the NPC. It will be important to determine the exact NPC binding sites that are critical for import and export to understand the details of the NPC.

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Makiko Koike, Shingo Kose, Maiko Furuta, Naoko Taniguchi, Fumihiko Yokoya, Yoshihiro Yoneda and Naoko Imamoto

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