Nuclear localization signal-independent and importin/karyopherin-independent nuclear import of β-catenin

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Background: Control of the nuclear localization of specific proteins is an important mechanism for regulating many signal transduction pathways. Upon activation of the Wnt signaling pathway, β-catenin localizes into the nucleus and interacts with TCF/LEF-1 (T-cell factor/lymphocyte enhancer factor-1) transcription factors, triggering activation of downstream genes. The role of regulated nuclear localization in β-catenin signaling is still unclear. β-catenin has no nuclear localization sequence (NLS). Although it has been reported that β-catenin can piggyback into the nucleus by binding to TCF/LEF-1, there is evidence that its import is independent of TCF/LEF-1 *in vivo*. Therefore, the mechanism for β-catenin nuclear localization remains to be established.

Results: We have analyzed β-catenin nuclear import in an *in vitro* assay using permeabilized cells. β-catenin docks specifically onto the nuclear envelope in the absence of other cytosolic factors. Docking is not inhibited by an NLS peptide and does not require importins/karyopherins, the receptors for classical NLS substrates. Rather, docking is specifically competed by importin-β/β-karyopherin, indicating that β-catenin and importin-β/β-karyopherin both interact with common nuclear pore components. Nuclear translocation of β-catenin is energy dependent and is inhibited by nonhydrolyzable GTP analogs and by a dominant-negative mutant form of the Ran GTPase. Cytosol preparations contain inhibitory activities for β-catenin import that are distinct from the competition by importin-β/β-karyopherin and may be involved in the physiological regulation of the pathway.

Conclusions: β-catenin is imported into the nucleus by binding directly to the nuclear pore machinery, similar to importin-β/β-karyopherin or other importin-β-like import factors, such as transportin. These findings provide an explanation for how β-catenin localizes to the nucleus without an NLS and independently of its interaction with TCF/LEF-1. This is a new and unusual mechanism for the nuclear import of a signal transduction protein. The lack of β-catenin import activity in the presence of normal cytosol suggests that its import may be regulated by upstream events in the Wnt signaling pathway.

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Background

A widespread mechanism for control of gene expression by signal transduction pathways involves the regulated compartmentalization of specific nuclear factors. Typically, such factors are excluded from the nucleus in the absence of signal, but accumulate in the nucleus upon activation of a particular pathway, where they modulate expression of specific genes.

A variety of mechanisms have been described that regulate the localization of such factors. For instance, the transcription factors NF-κB and Dorsal cannot constitutively enter the nucleus, because their nuclear localization sequences (NLS) are masked by a specific inhibitor protein, IκB or Cactus, respectively (see [1] for review). Signal-induced phosphorylation of IκB causes dissociation of the complex, leading to rapid import of NF-κB. Other factors are sequestered by cytoplasmic or membrane anchors until a signal releases this constraint.

Despite the large diversity of such regulatory mechanisms, all these signal-regulated factors are eventually imported into the nucleus using the classical import pathway. They possess an NLS (comprising one or two clusters of basic amino acids), which binds at the nuclear envelope through the so-called NLS receptor [2]. This NLS receptor comprises two cytosolic proteins: importin-α (also called α-karyopherin/srp1/NPI1) and importin-β (or β-karyopherin) [3–8] (see [9,10] for reviews). NLS binds to importin-α/α-karyopherin, which binds to importin-β/β-karyopherin, which in turn docks the complex to the nuclear pore by binding directly to nucleoporins [11–14]. The complex is

then translocated through the nuclear pore by an energy-dependent process involving the small soluble GTPase Ran/TC4 [15,16] and p10 (also called NTF2/pp15) [17,18].

Although the importin-α–importin-β complex functions as a rather generic NLS receptor, recent results have demonstrated the occurrence of specialized transport pathways for particular classes of essential nuclear factors [19–23]. Characteristically, these pathways are NLS independent but rely on importin-β-related molecules, which bind directly to the substrate without the contribution of importin-α. For instance, it has been found that the import of heterogeneous nuclear RNA-binding protein (hnRNP) A1 requires a sequence completely unrelated to the classical NLS that binds directly to transportin/Kap104p, an importin-β-like molecule [19,20]. It has also been reported recently that nuclear protein export is mediated by importin-β-like factors [24–27].

β-catenin, which mediates a late step of the Wnt/Wingless pathway [28–31], is a rather peculiar cytoplasmic signal transducer. Indeed, β-catenin was first identified as a component of the cell–cell adhesion complex, where it directly binds to the cytoplasmic tail of cadherin adhesion molecules [32]. Yet it appears that its signaling activity is independent of its function in adhesion [33,34]. On the other hand, there is now clear evidence that activation of the Wnt pathway leads to localization of β-catenin in the nucleus [34,35] and that the signaling activity of β-catenin involves its association with members of the high-mobility group (HMG) box family of transcription factors T-cell factor (TCF)/lymphocyte enhancer factor-1 (LEF-1)/pangolin [36–40]. Because β-catenin has no identifiable NLS, but was found to localize in the nuclei of TCF/LEF-1-overexpressing cells, it has been proposed that β-catenin is carried passively into the nucleus upon association with TCF/LEF-1 [36–38]. However, studies on Armadillo, the *Drosophila* β-catenin homolog, have shown that a mutant Armadillo defective in TCF binding still localizes to the nucleus [34,40]. This indicates that nuclear import of β-catenin in the *Drosophila* embryo is independent of TCF.

β-catenin and importins/karyopherins (α and β), as well as a number of other proteins, share interesting primary sequence similarities [5,9,41–43]. The central cores of these proteins consist of 8–13 related 42-amino-acid Armadillo repeats (from Armadillo, the *Drosophila* β-catenin homolog). These repeats are involved in many of the protein–protein interactions mediated by these molecules. Moreover, the Armadillo repeats of β-catenin are both necessary and sufficient for its nuclear localization [44], suggesting the possibility of functional similarities between β-catenin and importins.

Since regulation of β-catenin nuclear activity is clearly a key step in the Wnt signaling pathway, we have undertaken the molecular analysis of β-catenin nuclear import, which we have reconstituted here and dissected in an *in vitro* assay using permeabilized cells.

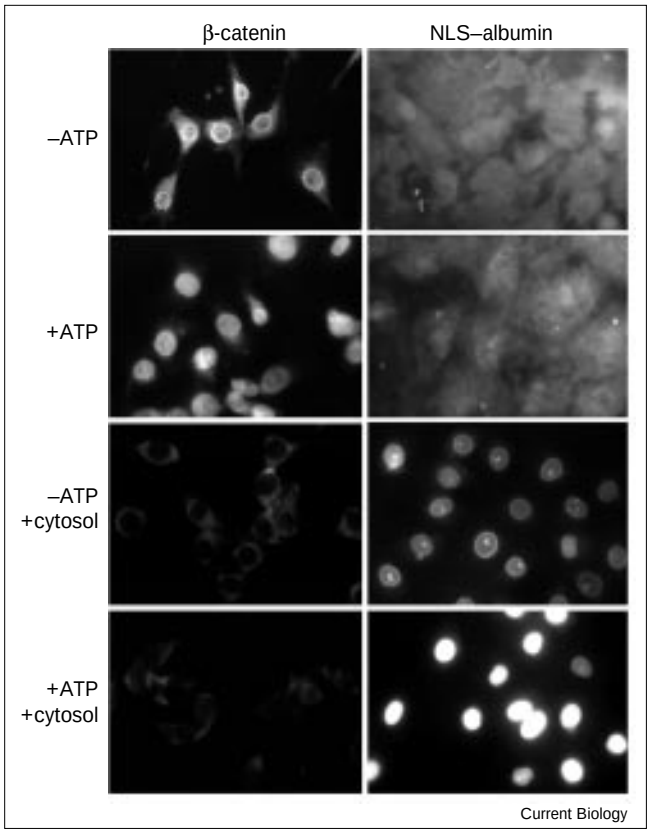
Results

β-catenin nuclear import in permeabilized cells

Digitonin-semipermeabilized cells have proven to be a powerful model system in which to study nuclear import [45]. Although most of the soluble cytosolic components are washed out during permeabilization, the nuclear envelope remains intact and nuclear import can be reconstituted using exogenous substrates. Using this assay, the nuclear import of NLS-containing molecules has been dissected into two distinct phases: docking to the nuclear pore, which is energy independent, and translocation through the pore, which requires ATP and/or GTP hydrolysis. Both steps are absolutely dependent on cytosolic factors (importins/karyopherins for docking and Ran for translocation). In the absence of these factors, both docking and translocation of substrate NLS–albumin (Figure 1) are negligible, but both steps can be restored by addition of exogenous cytosol (Figure 1) or purified factors (Figure 2a).

As a substrate for this assay, we have tested a recombinant β-catenin protein produced in baculovirus-infected Sf9 cells. This recombinant β-catenin was functional in signaling activity, as assessed by its ability to induce axis duplication at high frequency when injected directly into *Xenopus* embryos (approximately 50 ng protein/15 nl, data not shown). Incubation of permeabilized cells with recombinant β-catenin resulted in a prominent localization of β-catenin at the nuclear envelope in the absence of exogenous cytosol (Figure 1). Furthermore, β-catenin readily accumulated in the nucleus in the presence of an energy source (ATP, GTP and an energy-regenerating system). Addition of cytosol did not increase but rather inhibited docking and translocation (Figure 1, see also below). Although binding to other membranes and cytoplasmic structures was also observed, as might be expected from the many known interacting partners for β-catenin, the efficiency of nuclear docking and import was striking, particularly considering the low concentrations of β-catenin (approximately 0.2–1 μM) used in this assay. Docking at the nuclear envelope was observed both with fluorescein-labeled β-catenin (Figure 1) and with unlabeled β-catenin detected by indirect immunofluorescence (Figure 2), and similar results were obtained with both methods in the subsequent docking experiments. Only prelabeled fluorescein–β-catenin was used to study energy-dependent translocation into the nucleus, because unlabeled β-catenin was rather difficult to detect inside the nucleoplasm by immunofluorescence. Free access for the antibodies required permeabilization of the nuclei after fixation, probably resulting in poor retention of nuclear β-catenin (data not shown).

Figure 1



β-catenin docking and translocation in the absence of cytosolic factors. Permeabilized BRL cells were incubated with 0.5 μM fluorescein–β-catenin (left column) or 0.5 μM rhodamine–NLS–albumin (right column) in nuclear import buffer without ATP, GTP or cytosol (upper row); with ATP and GTP but no cytosol (second row); in the presence of cytosol (10 mg/ml) but no ATP or GTP (third row); and in the presence of ATP, GTP and cytosol (lower row). In –ATP conditions, apyrase was added to consume any trace of ATP. The +ATP conditions also contained GTP and an ATP-regenerating system (see Materials and methods).

β-catenin docking is NLS independent and is competed by importin-β

The observation that β-catenin can bind to the nuclear envelope in the absence of cytosolic factors suggests that β-catenin import is independent of the classical import pathway, which has a strict requirement for importins α and β. Nevertheless, cytosolic factors may not be completely depleted after permeabilization. In fact, a small pool of importin-β is known to remain associated with the nuclear pores [11]. Although unlikely, it is possible that such a pool could account for the observed accumulation of β-catenin at the nuclear envelope. In such a case, however, a third factor — an NLS-containing β-catenin-binding protein — would be required, since β-catenin has no NLS.

To test whether β-catenin might be imported by an NLS-dependent mechanism, we asked whether docking of β-catenin at the nuclear envelope could be inhibited by competition with an excess of NLS substrate. Docking of rhodamine–NLS–albumin is efficiently inhibited by an excess of unlabeled NLS–albumin (Figure 3), but not by a control with mutant NLS conjugated to albumin, which is not recognized by the NLS receptor [46]. We found, however, that the same excess of unlabeled NLS–albumin had no effect on the docking of β-catenin at the nuclear envelope (Figure 3).

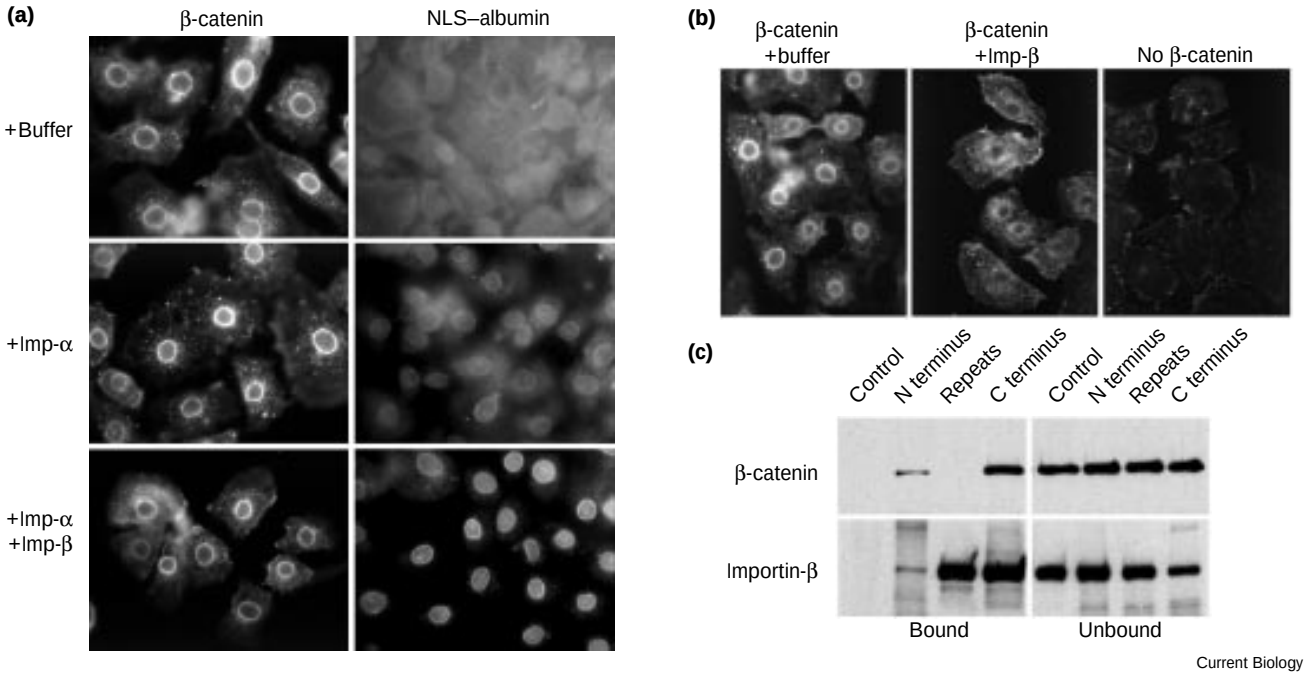
If β-catenin binding to the nuclear envelope occurred via importins α or β, binding should be enhanced by addition of exogenous importins. However, β-catenin binding was not further stimulated by concentrations of recombinant importins α and β that strongly increased NLS–albumin binding (Figure 2a). On the contrary, β-catenin docking was completely inhibited by low concentrations of importin-β (Figure 2b). This inhibition was highly specific: it was not observed upon addition of importin-α (Figure 2a), nor with large excesses (10–20 mg/ml) of a variety of unrelated proteins such as bovine serum albumin (BSA), ovalbumin, gelatin or casein (data not shown). Moreover, importin-β selectively inhibited β-catenin binding to the nuclear envelope, but not binding to other cellular structures, whose staining pattern remained unaltered (Figure 2b). Nuclear translocation of β-catenin in the presence of ATP and GTP was also strongly inhibited by importin-β (data not shown).

Importin-β is known to bind *in vitro* to a set of nuclear pore components (nucleoporins). Binding is thought to occur via multiple FXFG/GLFG (in the single-letter amino acid code, where X can be any amino acid) repeats conserved in nucleoporins [12,47,48]. Since β-catenin docking to the nuclear envelope does not require additional cytosolic factors and is competed by importin-β, we asked whether β-catenin could bind to nucleoporins. We compared the ability of importin-β and of β-catenin to bind to three recombinant fragments of yeast nucleoporin 1 (Nup1) fused to glutathione-*S*-transferase (GST). These fragments were, respectively, the amino terminus, the central region of FXFG repeats, and the carboxyl terminus, also containing six FXFG repeats as well as a nonrepeat region. β-catenin bound weakly to the amino terminus and strongly to the carboxyl terminus, but not to the central repeat region, whereas importin-β bound to the central repeats and to the carboxy-terminal fragment (Figure 2c). Although this experiment utilized yeast Nup1 rather than the physiological nucleoporin at the nuclear envelope, these data suggest that β-catenin can bind directly to nucleoporins.

Characteristics of β-catenin nuclear translocation

Wheat germ agglutinin is a classical inhibitor of nuclear translocation [45]. It blocks translocation by binding to

Figure 2



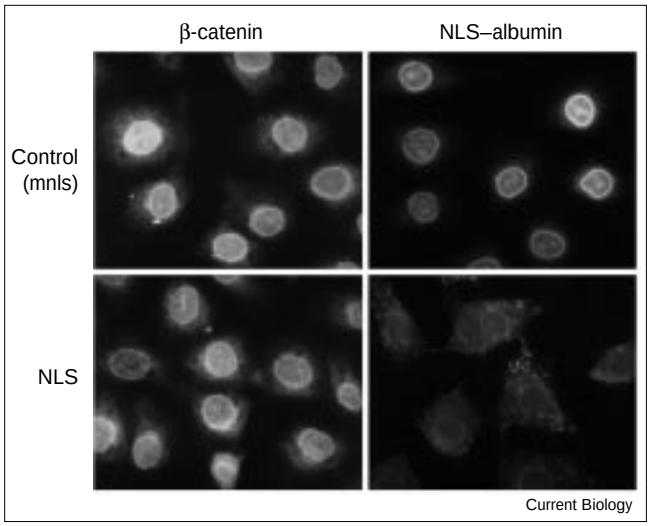
β-catenin docking is independent of importins and competed by importin-β. (a) β-catenin docking does not require importins. Cells were incubated with 0.2 μM unlabeled β-catenin (left column) or 0.5 μM rhodamine–NLS–albumin (right column) in nuclear import buffer alone (upper row); with 1.5 μM importin (Imp)-α (middle row); or with 1.5 μM importin-α and 70 nM importin-β (lower row). Unlabeled β-catenin was detected by immunofluorescence. Identical results were obtained with fluorescein–β-catenin (data not shown). (b) β-catenin docking is inhibited by importin-β. Permeabilized cells were incubated with 0.2 μM unlabeled β-catenin alone (left panel), or with 0.2 μM unlabeled β-catenin and 0.2 μM importin-β (middle panel). β-catenin was detected by immunofluorescence. Importin-β inhibited β-catenin staining completely and specifically at the nuclear envelope, but not β-catenin staining at cytoplasmic and membrane structures. Identical results were obtained with fluorescein–β-catenin (data not shown). The right panel shows control cells without exogenous β-catenin; a low background signal for endogenous β-catenin was detected, primarily at the cell surface. (c) *In vitro* binding of β-catenin and importin-β to Nup1. β-catenin and importin-β were incubated with three recombinant Nup1 fragments (N terminus, the amino terminus; C terminus, the carboxyl terminus; Repeats, the central FXFG repeat region) fused to GST and immobilized on glutathione–Sepharose beads (see Materials and methods). As a control, β-catenin and importin-β were incubated with glutathione-sepharose beads alone. Bound and unbound fractions (100% and 25%, respectively, loaded onto SDS gels) were analyzed by immunoblotting.

nucleoporins, which are *O*-glycosylated. We found that β-catenin import was also very effectively blocked by wheat germ agglutinin (Figure 4a), consistent with the involvement of nucleoporins in this process.

Translocation of NLS substrates requires GTP hydrolysis and is completely blocked by nonhydrolyzable GTP analogs [15,16]. We observed that the nonhydrolyzable nucleotide analog GMP–PNP (Figure 4b) and GTPγS (not shown) strongly inhibited β-catenin translocation, demonstrating that GTP hydrolysis is indeed required for β-catenin nuclear import. To observe this inhibition, however, preincubation of the permeabilized cells in the presence of the GTP analogs was required (data not shown), presumably in order to allow the endogenous GTPase to exchange bound nucleotides for the exogenous analogs. Note that preincubation by itself had no effect on docking and translocation of β-catenin.

The requirement for GTP hydrolysis during translocation of NLS substrates is at least partly due to the activity of the small GTPase Ran [9,49]. NLS translocation in the permeabilized cell assay is absolutely dependent on the addition of exogenous Ran [15,16], which is soluble and extensively depleted during permeabilization. On the other hand, although β-catenin translocation did require an energy source, it occurred in the absence of exogenous Ran (Figures 1, 5). Furthermore, addition of recombinant Ran protein did not stimulate, but rather partially inhibited, β-catenin translocation (Figure 5). Control experiments showed that the same recombinant Ran protein was very effective in translocation of NLS–albumin (data not shown). There is a possibility, however, that a pool of Ran, or of some Ran-like GTPase, is still present in permeabilized cells, for instance at the nuclear pore or inside the nucleus, in sufficient amounts to trigger β-catenin translocation. To determine whether Ran, or a Ran-like

Figure 3



β-catenin docking is not competed by NLS–albumin. Cells were incubated with 0.5 μM fluorescein–β-catenin (left column) or 1 μM rhodamine–NLS–albumin (right column) in the presence of 20 μM unlabeled mutant NLS (mnls) conjugated to albumin (upper panels) or unlabeled wild-type NLS conjugated to albumin (lower panels). Mutant NLS-albumin was used as a control, since it is deficient in nuclear docking and import. With rhodamine–NLS–albumin (right panels), exogenous cytosol (10 mg/ml) was included in the assay as a source of importins for docking.

factor, was indeed involved in β-catenin translocation, we tested the effect of a dominant-negative form of Ran, RanGln69→Leu (RanQ69L), which preferentially binds, but does not hydrolyze, GTP [50–52]. RanQ69L causes a complete immediate inhibition of cytosol-driven import of NLS-albumin ([51,52]; data not shown). When β-catenin and RanQ69L were added simultaneously to the cells, only a weak inhibition of β-catenin import was observed (data not shown). However, RanQ69L blocked β-catenin translocation very efficiently when presented to the cells during a preincubation period (Figure 5), suggesting, therefore, that GTP hydrolysis by Ran or a related factor is indeed required for β-catenin import. The fact that a preincubation period was necessary suggests that the dominant-negative Ran probably had to displace an endogenous factor from the nuclear pore machinery.

Cytosol contains inhibitory activities for β-catenin import

As mentioned above, addition of cytosol clearly inhibited β-catenin docking and import. Inhibition was not due to degradation of β-catenin, as assessed by immunoblotting (data not shown). Since importin-β could efficiently block β-catenin import in our assay, we tested whether it may account for the inhibitory activity of the cytosol (Figure 6). Cytosol was fractionated by ion-exchange chromatography, and fractions were tested for inhibitory activity on β-catenin import, as well as for the presence of importin-β. As shown in Figure 6, the main inhibitory activity did not cofractionate with importin-β (fractions 5 and 6), indicating that other unidentified cytosolic factor(s), which may include other importin-like factors, are involved. Inhibition by fractions 2 and 3 appeared qualitatively different from the selective inhibition of nuclear membrane

Figure 4

Characterization of β-catenin translocation using confocal microscopy: effects of wheat germ agglutinin and GTP analogs. (a) Translocation is inhibited by wheat germ agglutinin. Cells were incubated with fluorescein–β-catenin in the absence of ATP and GTP (left panel), in the presence of ATP and GTP (middle panel), or in the presence of ATP, GTP and wheat germ agglutinin (WGA, right panel), and examined by confocal microscopy. (b) Translocation is inhibited by GMP–PNP. Cells were incubated with fluorescein–β-catenin in the absence of ATP and GTP (left panel), in the presence of ATP and GTP (middle panel), or in the presence of ATP, GTP, and 1 mM GMP–PNP (right panel), and examined by confocal microscopy. Note that to observe inhibition of translocation, the cells were first incubated for 20 min (in the presence or absence of GMP–PNP) before incubation with β-catenin.

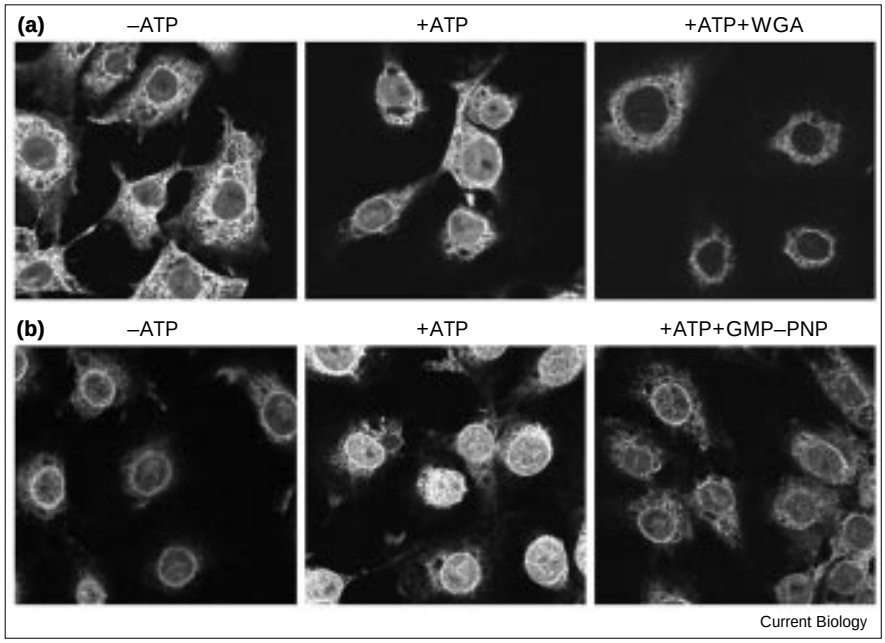
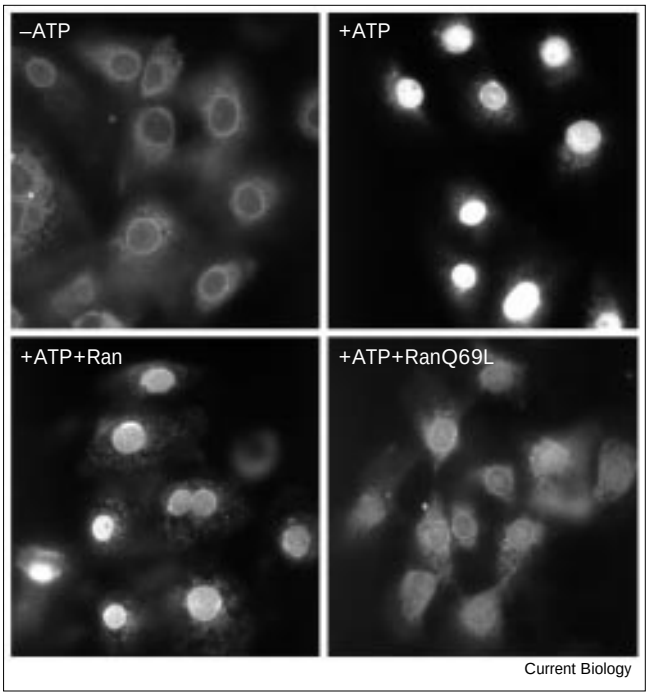


Figure 5



Effects of Ran GTPase and dominant negative Ran on β-catenin translocation. Cells were incubated with fluorescein–β-catenin in the absence of ATP and GTP (top left panel), in the presence of ATP and GTP (top right panel), in the presence of ATP, GTP, and 6 μM Ran (bottom left panel), or in the presence of ATP, GTP, and 6 μM RanQ69L (bottom right panel). Note that the cells were first preincubated for 20 min in all conditions, in the presence or absence of Ran or RanQ69L, before incubation with β-catenin. Exogenous Ran did not increase, but rather partially inhibited β-catenin translocation, under conditions where it optimally stimulated importin-mediated NLS–albumin translocation (data not shown). RanQ69L strongly inhibited β-catenin translocation when present during a preincubation period. RanQ69L was not effective without preincubation (data not shown).

docking by importin-β because these fractions inhibited both nuclear membrane and cytoplasmic staining. Fraction 4 inhibited binding to the nuclear membrane without decreasing cytoplasmic staining. Fractions 1 and 6, which contained high amounts of total protein, had no affect on β-catenin docking at the nuclear envelope, nor did large excesses (10–20 mg/ml) of a variety of unrelated proteins, such as BSA, ovalbumin, gelatin, or casein (data not shown). Therefore, inhibition of nuclear docking and import by the cytosol cannot be attributable to either the presence of importin-β or high levels of protein in the sample causing nonspecific inhibition.

Discussion

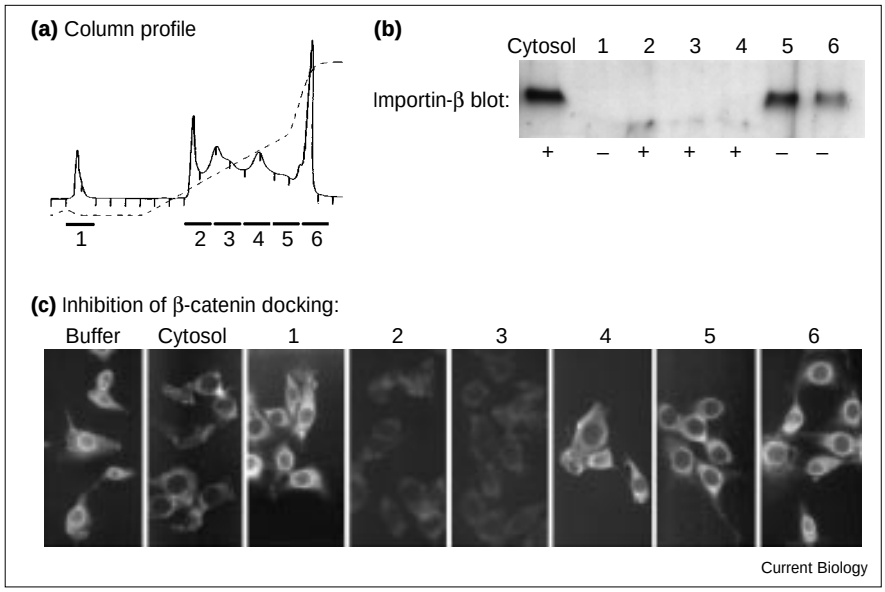
In this study, we have analyzed the mechanism of nuclear import of β-catenin using an established *in vitro* nuclear import assay. β-catenin is imported into the nucleus despite the fact that it lacks a readily identifiable NLS sequence. We have now found that β-catenin is imported directly by an NLS-independent and importin/karyopherin-independent mechanism.

The conclusion that β-catenin import is NLS independent is based on the following observations. Firstly, β-catenin binds directly to the nuclear envelope and can be translocated in the absence of additional cytosolic factors. Secondly, docking of β-catenin is not enhanced by addition of recombinant importins, the receptors that recognize NLSs and dock NLS-containing proteins to the nuclear envelope. Thirdly, docking of β-catenin is not competed by NLS peptide, which does block docking of NLS-containing proteins. Thus, β-catenin seems to bypass the initial receptor-dependent event of the classical import pathway and dock directly at the nuclear envelope.

Although independent of importins, the mechanism used for β-catenin docking appears to overlap with the classical NLS import pathway. Indeed, β-catenin binding to the nuclear envelope was specifically competed by importin-β, suggesting that either common or closely adjacent docking sites are involved. Furthermore, β-catenin, like importin-β, was found to bind *in vitro* to a yeast recombinant nucleoporin, Nup1. Although Nup1 is not the physiological docking site for either β-catenin or importin-β, it contains numerous FXFG consensus repeats that are thought to be involved in the docking of importin-β to the nuclear pore and its subsequent translocation into the nucleus [8,12,47]. The ability of β-catenin to bind to fragments of Nup1 and the competition of its binding to the nuclear envelope by importin-β suggest that it docks to the nuclear envelope by binding to nucleoporins. A detailed comparison of the physiological binding sites for β-catenin and importin-β at the nuclear pore is not yet possible, since the identity of the nucleoporin responsible for the docking of importin-β is not known with certainty and is definitely not yet known for β-catenin. Also, although the observed competition between importin-β and β-catenin for binding to the nuclear envelope can be taken as evidence of a common feature for both pathways, such competition may not occur physiologically during nuclear import in the cell. Indeed, the enormous capacity and speed of import through a nuclear pore [10] clearly allows parallel pathways to coexist (e.g. those mediated by importin-β and transportin [52]).

Translocation of β-catenin through the nuclear pore exhibits the same basic features as the classical NLS-dependent import mechanism. Translocation is energy dependent; is blocked by wheat germ agglutinin, which binds to *O*-linked sugars on nucleoporins, consistent with requirement for interaction with nucleoporins during transport; and is inhibited by nonhydrolyzable GTP analogs, indicating the involvement of a GTPase. Thus, β-catenin resembles importin-β by its ability to bind directly

Figure 6



Inhibition of β-catenin docking by cytosolic activities distinct from importin-β. Crude cytosol was fractionated on an anion exchange column, fractions were pooled, concentrated and tested for the presence of importin-β and for inhibition of β-catenin docking. **(a)** Elution profile of cytosol on a HiTrapQ anion exchange column (see Materials and methods). Dotted line: NaCl gradient (from 20 to 750 mM). **(b)** Importin-β immunoblot of total cytosol and of pooled fractions 1–6, showing that importin-β elutes in fractions 5 and 6. **(c)** Inhibition of β-catenin docking by cytosol fractions. Permeabilized cells were incubated with fluorescein-labeled β-catenin in import buffer, without ATP or GTP, mixed with crude cytosol or with fractions 1–6. The inhibitory activity elutes in fractions 2, 3 and 4. Note that fractions 2 and 3 inhibited both nuclear membrane staining and cytoplasmic staining, whereas fraction 4 primarily inhibited nuclear membrane staining.

to the nuclear envelope, and by its translocation by a related, if not the same, pathway.

However, β-catenin translocation seems to differ from the classical pathway with respect to the requirement for the cytosolic GTPase Ran. While Ran is absolutely necessary for translocation of the NLS–importin complex, β-catenin was imported quite well in the absence of any exogenous factor. In this regard, it is interesting that β-catenin seems to behave similarly to transportin, which can also be translocated into the nuclei in the absence of exogenous Ran [20]. Thus, β-catenin import must rely on some GTPase activity still present in permeabilized cells, perhaps one remaining bound to the nuclear pores. This activity could be due to residual Ran, but the data are not completely consistent with this explanation. Experiments with NLS substrates indicate that endogenous Ran activity is extensively washed away during permeabilization ([4]; data not shown). Also, if β-catenin import utilizes Ran, we would have expected some stimulation of β-catenin translocation by addition of exogenous Ran, but instead, recombinant Ran partially inhibited import of β-catenin. On the other hand, β-catenin translocation was very sensitive to inhibition by dominant-negative mutant Ran, RanQ69L, which is presumed to act as a specific antagonist for Ran. These results suggest three obvious possibilities. Firstly, Ran might indeed be the GTPase involved in translocation of β-catenin, but an additional component would be limiting in permeabilized cells. The p10 protein appears to be involved in translocation of NLS-containing proteins [17,18], but adding it had no effect on β-catenin import (data not shown). It is still possible that other soluble factors are specifically required for β-catenin translocation. Secondly, β-catenin import may utilize another Ran-like GTPase. Both Ran and RanQ69L may then compete with this GTPase and exhibit dominant-negative activities in our assay. Thirdly, it is conceivable that Ran or a Ran-like GTPase may be involved in the re-export of β-catenin rather than its import. Stimulation of re-export by exogenous Ran could then result in a decreased net nuclear accumulation of β-catenin.

Importin-β/β-karyopherin and transportin are members of a growing family of importin-β-like proteins that all appear to be involved in nuclear transport [27]. Interestingly, many of these proteins, as well as importin-α/α-karyopherin, contain Armadillo repeat sequences [5,9,41,42]. Until now, there has been no known functional relationship between importins and other proteins that contain Armadillo repeats, in particular β-catenin, Armadillo and the related protein plakoglobin, all of which are known to function in cell–cell adhesion and signal transduction [28,29]. Yet the findings that these three junctional proteins also accumulate in the nucleus [33–35,44,53], and that the Armadillo repeats alone are sufficient for nuclear localization of β-catenin [44], suggest the intriguing possibility of a functional relationship between all Armadillo repeat-containing proteins. From our findings on the molecular mechanism of β-catenin import we now find clear evidence for a functional relationship between β-catenin and importin-β. It is tempting to speculate that the capacity for nuclear envelope docking and for import may emerge as a general property of the Armadillo repeat-containing proteins.

It has been proposed that β-catenin is imported into the nucleus via its physical interaction with the TCF/LEF-1

transcription factor, which contains a consensus sequence for an NLS and can thus probably be imported into the nucleus by the classical import pathway. In cell transfection experiments, nuclear import of β-catenin has been shown to be stimulated by coexpression of TCF/LEF-1 [37,38]. However, these two proteins may not necessarily be imported together into the nucleus under physiological circumstances. Indeed, the nuclear import of *Drosophila* Armadillo was found to occur independently of its interaction with *Drosophila* TCF. A mutant form of Armadillo lacking the fifth Armadillo repeat fails to bind to *Drosophila* TCF (and therefore lacks signaling activity), but accumulates in embryonic nuclei in response to Wingless (Wnt) signaling [34,40]. Our findings reported here provide an explanation for how this mutant Armadillo could undergo nuclear import, that is by an NLS-independent and importin-independent (and therefore TCF/LEF-1-independent) mechanism. Indeed, it might make sense that β-catenin would be imported separately from TCF/LEF-1 under physiological circumstances. β-catenin is normally not localized in the nuclei of most cells, but is triggered to enter nuclei during the transduction of a signal initiated by the Wnt (or similar) pathway. TCF/LEF-1 is typically a nuclear protein and may be imported constitutively.

We propose that the role of the NLS-independent and importin-independent mode of β-catenin nuclear import is to provide a mechanism for developmental and physiological regulation of its signaling activity. In the simplest model, this import step would allow β-catenin to associate with TCF/LEF-1 that is already present in the nucleus and perhaps prebound to DNA. This would be consistent with reports that β-catenin acts as a transcriptional activator when it is bound to TCF/LEF-1 at specific DNA sequences. An intriguing alternative model is that β-catenin could also act as a specialized regulated importin to carry other proteins into the nucleus in response to specific upstream signals, although there is no evidence so far for a β-catenin-mediated import of other nuclear factors.

An important problem will be to understand how the β-catenin nuclear import mechanism is regulated by the Wnt signaling pathway. We propose that the observed inhibition of β-catenin import by cytosol may be a reflection of this regulation. Cytoplasmic retention of nuclear proteins, accomplished by a variety of different mechanisms, is a well-known mechanism for signal-dependent regulation of nuclear import. The inhibition of β-catenin import by cytosol seems to be specific, as it does not result simply from the presence of high protein concentration. Moreover, the inhibitory activity is not due to competition by importin-β present in the cytosol, since it fractionates as a peak on Mono-Q columns distinct from the fractions containing importin-β. The identities of the inhibitory factors are unknown, but may include other importin-like factors. The fact that cytosol affects binding of β-catenin both to the nuclear membrane and to other cytoplasmic structures suggests either that the inhibitory activities result from more than one factor or that an inhibitory factor acts at the level of β-catenin, modifying it in such a way as to affect its binding to more than one cellular component. To test the hypothesis that inhibition by cytosol reflects the physiological regulation of nuclear import, it will be important to identify the inhibitory factor(s) and to determine the conditions that overcome this inhibition and allow β-catenin import in response to upstream signaling events.

Signaling by the Wnt pathway could regulate β-catenin import in several different ways. One prevailing model in the literature is that accumulation of β-catenin to high levels in the cytosol in response to upstream signaling events is sufficient to transduce the signal to the nucleus [30]. Wnt signaling has been shown to slow the rate of turnover of β-catenin and Armadillo and to result in their cytosolic accumulation [54–57]. However, signaling is also associated with changes in β-catenin and Armadillo phosphorylation [55,57] as well as changes in the phosphorylation of β-catenin-associated proteins, such as the adenomatous polyposis coli (APC) tumor suppressor protein [58]. In addition to regulating the levels of β-catenin, such post-translational modifications could alter the intrinsic capacity of β-catenin to undergo nuclear import. Either mechanism could potentially entail a role for a cytosolic inhibitor of nuclear import. Thus, further studies on the mechanism of β-catenin nuclear import and the role of cytosolic inhibitors in the regulation of import may provide key elements for our understanding of the mechanism of signal transduction by the Wnt pathway.

Conclusions

Our data show that β-catenin is imported into the nucleus by binding directly to the nuclear pore machinery, similarly to importin-β/β-karyopherin or other importin-β-like import factors. This pathway accounts for the fact that β-catenin localizes to the nucleus without an NLS and independent of its interaction with TCF/LEF-1. Furthermore, the similarity in the mechanism of nuclear import between β-catenin and importin-β-like molecules suggests a functional relationship between Armadillo repeat-containing proteins. The lack of β-catenin import activity in the presence of normal cytosol is consistent with the hypothesis that this step may be regulated by upstream events in the Wnt signaling pathway.

Materials and methods

Recombinant proteins and reagents

Amino-terminally 6×His-tagged full-length *Xenopus* β-catenin [59] was expressed in Sf9 cells using a baculovirus (gift of R. Kypta, UCSF) expression system (Clontech) and purified as follows: Cells (4×10^6/ml) were lysed in 0.1% NP-40, 4 mM imidazole, 500 mM NaCl, 20 mM Tris HCl pH 7.9, 1 mM mercaptoethanol and a cocktail of protease inhibitors (1 mM PMSF, 0.2 μg/ml leupeptin, 0.4 μg/ml

aprotinin, 10 μg/ml antipain, 50 μg/ml benzamidine). The lysate was centrifuged for 30 min at 100,000 × *g*, and the supernatant was incubated with Ni-NTA beads (Qiagen; 1 ml beads/100 ml lysate) for 3 h at 4°C. The beads were then washed with 10 mM imidazole, 100 mM NaCl, 20 mM Tris HCl pH 7.9, 5% glycerol, and 6×His–β-catenin was eluted with 500 mM imidazole, 100 mM NaCl, 20 mM Tris HCl, pH 7.9, 5% glycerol. The purity of the β-catenin fractions was assessed by Coomassie staining and western blotting after SDS–PAGE. Purified β-catenin was labeled with FITC or carboxyfluorescein succimidyl ester (Molecular Probes) as suggested by the manufacturer. Coupling was estimated to yield a molar ratio of fluorescein:β-catenin of 1 to 2. Bacterial recombinant *Xenopus* importins α and β, His-tagged human Ran, and dominant-negative RanQ69L were generous gifts of D. Görlich (University of Heidelberg). Clones for bacterial recombinant β-karyopherin (used for *in vitro* binding experiments, see Figure 2c) and for p10 were a gift of G. Blobel (Rockefeller University), and both proteins were expressed and purified as published [12,17]. NLS peptides of SV40 T antigen (CYTPPKKKRKV) or mutant NLS peptides (CYTPPKTKRKV) were coupled to human serum albumin, and rhodamine-labeled NLS–human serum albumin was prepared as described [46]. Constructs for recombinant yeast Nup1 fragments fused to GST were a gift from L. Davis (Duke University). Antibodies used in these experiments were: rabbit anti-amino-terminal β-catenin [60] and rabbit anti-β-karyopherin (gift of G. Blobel).

Nuclear import assay

The nuclear import assay using permeabilized cells was modified from previously published protocols [4,45,46]: BRL cells were grown to subconfluency on glass coverslips, permeabilized at 4°C with 35 μg/ml digitonin in nuclear import buffer (NIB), washed with 10 mg/ml BSA in NIB, and incubated for 20–60 min at room temperature on a 20 μl drop containing the various reagents equilibrated in NIB, as well as 10 mg/ml BSA. NIB contained: 250 mM sucrose, 100 mM potassium acetate, 20 mM Hepes KOH, pH 7.4, 2 mM magnesium acetate, 2 mM DTT, 1 mM EGTA, and protease inhibitors (10 μg/ml leupeptin, 5 μg/ml chymostatin, 1 μg/ml elastinal). ATP-depleted samples contained 100 U/ml apyrase. For +ATP conditions, 1 mM ATP, 0.2 mM GTP, 10 mM creatine phosphate, and 20–40 U/ml phosphocreatine kinase were present during the incubation. Unless otherwise stated, reagents were used at the following concentrations: unlabeled β-catenin and fluorescein–β-catenin, ~0.2–2 μM; rhodamine–NLS-albumin, ~1 μM; importin-α, 1.5 μM; importin-β, 70–150 nM; Ran, 6 μM; RanQ69L, 6 μM; rhodamine-labeled wheat germ agglutinin, 0.1 mg/ml; GTPγS and GMP–PNP, 1 mM. At the end of the reaction, the cells were fixed with 3% paraformaldehyde and either mounted and observed directly or processed for immunofluorescence. Cells were observed under a Zeiss Axioplan epifluorescence microscope or using a BioRad 600 laser-confocal mounted on an Axiovert Zeiss microscope.

In vitro *binding assay*

Lysates from bacteria expressing GST–Nup1-amino-terminal region (amino acids 5–381), GST-Nup1-repeats (amino acids 433–816) or GST-Nup1-carboxy-terminal region (amino acids 778–1076) were incubated with glutathione–Sepharose (Pharmacia) for 15 min at 25°C (10 μg GST-fusion protein/10 μl packed beads). The beads were then washed extensively in PBS and resuspended as a 50% slurry in binding buffer (250 mM NaCl, 20 mM Hepes pH 7.0, 5 mM EDTA, 0.5 mM DTT, 0.1% Tween 20): 30 μl slurry were incubated with 4 μg β-catenin or 6 μg β-karyopherin in a total volume of 150 μl binding buffer for 30 min at room temperature tumbling end over end. After a 30 sec spin at 2,000 × *g*, the supernatant was collected (unbound fraction) and the beads were washed five times with 0.5 ml binding buffer. Aliquots of bound and unbound fractions were analyzed by SDS–PAGE and immunoblotting.

Fractionation of cytosol by anion exchange chromatography

Crude cytosol from *Xenopus* oocytes, prepared as published [46], was dialyzed against 20 mM NaCl, 50 mM Tris HCl pH 7.4. Samples containing about 20 mg protein were loaded on a 1 ml HiTrapQ column (Pharmacia). The column was eluted with 10 ml of 20 mM NaCl, 50 mM Tris HCl pH 7.4, followed by a 10 ml gradient from 20 mM to 375 mM NaCl, and by a wash at 750 mM NaCl (see Figure 5a). Farctions (1 ml) were collected and pooled together into six fractions as indicated in Figure 5. Each of the six fractions were concentrated fourfold, equilibrated in nuclear import buffer by dialysis and tested for inhibition of β-catenin import. Fractions (8 μl, i.e. ~0.2–1 mg protein/ml) were used in a 20 μl reaction.

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