Signalling activity of β -catenin targeted to different subcellular compartments

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β -Catenin plays a dual role as an adhesion molecule in adherens junctions at the plasma membrane and as a key intermediate in the canonical Wnt signalling pathway. The cytosolic soluble pool of β -catenin, involved in the transmission of the Wnt signal, is normally subjected to rapid protein degradation. On activation of the Wnt cascade, β -catenin becomes stabilized and then translocates into the nucleus where it co-activates transcription factors of the TCF (T-cell factor)/LEF (lymphoid enhancer factor) family. The expression of plasma membrane-targeted forms of β -catenin has been shown to also activate TCF/LEF-dependent transcription and different mechanisms have been put forward. In the present study, we have undertaken a systematic analysis of the signalling capability of non-degradable forms of β -catenin targeted to different cellular compartments. β -Catenin targeted to the plasma membrane activated transcription to a greater extent compared with non-targeted β -catenin, and led to a marked

stabilization of cytosolic soluble β -catenin. These effects were independent of the competition with endogenous β -catenin for binding to E-cadherin at the plasma membrane, since targeting non-degradable β -catenin to other cellular compartments, i.e. the outer mitochondrial membrane and the endoplasmic reticulum membrane, also resulted in the accumulation of cytosolic wild-type β -catenin and activation of β -catenin-dependent signalling. In contrast, nuclear-targeted β -catenin was without significant effect on cytosolic wild-type β -catenin and did not activate transcription. Our results suggest that cytosolic accumulation of β -catenin is a prerequisite for the activation of TCF/LEF-dependent transcription in the nucleus.

Key words: β -catenin, subcellular localization, transcriptional activity, Wnt signalling.

INTRODUCTION

β -Catenin is a bifunctional protein and exists in two separate pools in the cell. β -Catenin localized at the plasma membrane is bound to E-cadherin and α -catenin within adherens junctions. The cytosolic soluble pool of β -catenin is involved in the transmission of the canonical Wnt signal from the cell membrane to the nucleus. Structurally, β -catenin is composed of 12 central Arm (armadillo) repeats, flanked by an N- and a C-terminal domain [1]. The C-terminus contains the DNA-transactivation domain, whereas the N-terminus contains a number of conserved serine and threonine residues, which, when phosphorylated, target cytosolic β -catenin for ubiquitination and rapid protein degradation. The kinases responsible for the phosphorylation event have been identified as CK1 and GSK-3 (glycogen synthase kinase-3) [2,3] and they exist in a complex that also includes Axin and the APC (adenomatous polyposis coli) protein [4]. The current consensus view of the canonical Wnt signalling pathway (note that alternative mechanisms have been proposed recently [5]) is that activation of the Wnt cascade after the binding of the Wnt ligand to the frizzled/LRP (LDL-receptor-related protein) co-receptor leads to the inhibition of GSK-3-dependent phosphorylation of β -catenin [6]. Inhibition of GSK-3 activity is believed to be mediated by the proteins dishevelled and/or FRAT (frequently rearranged in advanced T-cell lymphomas) [7,8]. As a result of GSK-3 inhibition, cytosolic β -catenin can accumulate and translocate into the nucleus where it co-activates transcription factors of the TCF (T-cell factor)/LEF (lymphoid enhancer factor) family, resulting in the transactivation of genes such as cyclin D1 or c-Myc [9–11].

Recently, using a *Drosophila* system, it has been shown that non-degradable forms of the *Drosophila* homologue of β -catenin, Arm, are more efficient in activating gene transcription when targeted to the plasma membrane compared with the expression of non-targeted protein [12]. In addition, Arm targeted to the nucleus could not activate transcription. The effect of plasma membrane-targeted Arm was not dependent on endogenous Arm, as membrane-targeted β -catenin also activated transcription in arm^{XM19} mutants. These mutants express Arm that lacks the C-terminal domain, including the transcriptional activation domain. The authors suggested that rather than acting as a nuclear co-activator of TCF/LEF transcription factors, β -catenin transduces the Wnt signal by mediating nuclear export of TCF/LEF proteins, which in the absence of β -catenin, can act as repressors, or by inducing cytosolic activation of TCF transcription factors.

The results of the study by Chan and Struhl [12], in the context of earlier reports on the signalling ability of different membrane-targeted forms of β -catenin [14–16], have been discussed in detail by Bienz and Clevers [13]. On the basis of conclusions of a number of studies [14–16], the authors argue that the signalling ability of plasma membrane-targeted Arm/ β -catenin is due to an increase in endogenous soluble β -catenin, since the arm^{XM19} mutant used in [12] retains some transcriptional activity [17]. In addition, there is overwhelming evidence to suggest that stimulation of TCF/LEF-dependent transcription through activation of Wnt signalling correlates with the nuclear translocation of β -catenin [13]. However, a number of questions have still not been addressed, including whether the signalling ability of non-nuclear β -catenin is confined to proteins targeted to the plasma membrane and why

Abbreviations used: APC, adenomatous polyposis coli; Arm, armadillo; GSK-3, glycogen synthase kinase-3; HA, haemagglutinin; HEK-293 cells, human embryonic kidney-293 cells; LEF, lymphoid enhancer factor; NLS, nuclear localization sequence; TCF, T-cell factor.

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Arm targeted to the nucleus appears to be incapable of activating TCF/LEF-mediated transcription.

In this study, using a mammalian expression system, we have systematically analysed the signalling ability of non-degradable β -catenin targeted to different cellular compartments. The results indicate that targeting of β -catenin to different intracellular membranes increases the cytosolic β -catenin concentration and activates TCF/LEF-dependent transcription, suggesting that this effect is due to inhibition of the degradation of cytosolic protein. In contrast, nuclear expression of β -catenin does not lead to a significant increase in cytosolic wild-type protein and does not activate TCF/LEF-dependent transcription, suggesting that initially β -catenin must accumulate in the cytosol before being able to elicit signalling activity in the nucleus.

EXPERIMENTAL

Plasmid constructs, mutagenesis and transfection of HEK-293 cells (human embryonic kidney-293 cells)

The expression vector for human V5- β -catenin was from Invitrogen (Carlsbad, CA, U.S.A.) and for human TCF4 from Upstate (Lake Placid, NY, U.S.A.). ErbB2 cDNA was a gift from Dr John Timms (Ludwig Institute for Cancer Research, London W1W 7BS, U.K.). Bcl2 cDNA was cloned from a human foetal brain cDNA. The S45A (Ser⁴⁵ → Ala) mutant of V5- β -catenin was generated using the Stratagene site-directed mutagenesis kit. All open reading frames were cloned into the pcDNA3 expression vector (Invitrogen) and transfected into sub-confluent HEK-293 cells using Eugene 6 reagent (Roche) or Gene-Juice (Novagen) according to the manufacturer's instructions. Cells were assayed for reporter activity, 48 h after transfection, fixed for immunocytochemistry or cell lysates were prepared for Western-blot analysis. DNA constructs were generated as follows. cat[S45]A: the full-length coding sequence of β -catenin, including the C-terminal V5-tag, was PCR-amplified from S45A-mutant V5- β -catenin-pcDNA3.1/GS (Invitrogen) with flanking *KpnI* and *XbaI* restriction sites and ligated into pcDNA3. Erb-cat[S45A]: the sequence encoding amino acids 1–750 of ErbB2, including the extracellular and transmembrane domains, but excluding the intracellular tyrosine kinase domain, was PCR-amplified and ligated into the *HindIII* and *KpnI* sites of pcDNA3. The full length sequence of S45A- β -catenin, including a C-terminal V5 tag and excluding the start codon, was then fused in-frame to ErbB2(1–750), using the *KpnI* and *XbaI* sites. cat[S45A] Δ CT: the sequence encoding residues 1–666 of S45A- β -catenin was PCR-amplified, including a C-terminal V5 tag, and cloned into the *KpnI* and *XbaI* sites of pcDNA3. Erb-cat[S45A] Δ CT: as ErbB2-cat[S45A], except that residues 2–666 of S45A- β -catenin, including a C-terminal V5 tag, were fused to the ErbB2 gene lacking the tyrosine kinase domain. cat[S45A] Δ CT-FAR: a farnesylation signal, consisting of the 20 C-terminal residues of c-Ha-Ras, was fused in-frame to the C-terminus (i.e. the V5 tag) of cat[S45A] Δ CT. Erb-cat[S45A]R10: as Erb-cat[S45A], except that residues 2–525 of S45A- β -catenin, including a C-terminal V5 tag, were fused to the ErbB2 gene lacking the tyrosine kinase domain. cat[S45A]OMM: the sequence encoding amino acids 492–520 of human monoamine oxidase B [18], generated by PCR, was fused in-frame to the C-terminus (i.e. the V5 tag) of cat[S45A]. cat[S45A]Bcl2-TM: the full length sequence of S45A- β -catenin, including a C-terminal V5 tag, but lacking a stop codon, was PCR-amplified and cloned into the *KpnI* and *BamHI* sites of pcDNA3. The sequence encoding the transmembrane domain of human Bcl2 (residues 211–239) was then amplified from Bcl2 cDNA and

ligated into the *BamHI* and *XbaI* sites. cat[S45A]nucl: two copies of the nuclear localization sequence of SV40 large T antigen (encoding for DPKKKRKVDPKKKRKV) were generated by PCR and attached at the N-terminus (between the start codon and residue 2) and the C-terminus (between the last residue and V5 tag) of cat[S45A]. dnTCF4: TCF4, excluding amino acids 1–31, which form the β -catenin-binding domain, was PCR-amplified, including a start codon at the N-terminus and a V5 tag (to confirm protein expression) at the C-terminus, and cloned into the *KpnI* and *XbaI* sites of pcDNA3. wt HA (haemagglutinin)- β -catenin: the full length sequence of wild-type β -catenin, including a C-terminal HA tag, was PCR-amplified from V5- β -catenin-pcDNA3.1/GS, and cloned into the *KpnI* and *XbaI* sites of pcDNA3. Sequences were confirmed by DNA sequencing. To generate a cell line with tetracycline-inducible expression of Erb-cat[S45A], the T-Rex system (Invitrogen) was used. The Erb-cat[S45A] coding sequence was transferred into pcDNA4/TO using the *HindIII* and *XbaI* sites. Erb-cat[S45A]-pcDNA4/TO was transfected into T-Rex-HEK-293 cells and single clones were isolated using zeocin selection. Tetracycline-dependent protein expression was confirmed by Western-blot analysis.

Immunoblotting and immunocytochemistry

For immunoblotting, cells were washed with ice-cold PBS and then lysed in RIPA lysis buffer (150 mM NaCl/50 mM Tris, pH 7.5/2 mM EDTA/0.5 % Nonidet P40/0.1 % mercaptoethanol + protease inhibitor cocktail; Roche). Equal amounts of protein from total lysates were used for Western-blot analysis. For measurements of cytosolic β -catenin by immunoblotting, a hypotonic lysis buffer was used as described in [19]. Hypotonic lysates were precleared by centrifugation before use. Antibodies were obtained from the following sources: monoclonal Anti- β -catenin (BD Biosciences, San Diego, CA, U.S.A.), monoclonal Anti-V5 (Serotec, Oxford, U.K.) and rat monoclonal Anti-HA (clone 3F10; Roche, Indianapolis, IN, U.S.A.). For immunocytochemistry, cells were fixed using paraformaldehyde and, after blocking with 5 % normal goat serum, incubated with V5-antibody and secondary FITC-conjugated anti-mouse IgG. For mitochondrial staining, cells were incubated with 1 μ M MitoTracker Red (Molecular Probes, Eugene, OR, U.S.A.) for 1 h before fixing the cells. Nuclei were labelled using DAPI.

Topflash reporter assays

TCF/LEF-dependent transcription was measured using the Topflash reporter construct (Upstate) as described previously [20]. Results shown are representative of at least three independent experiments (means $\pm$ S.D.).

RESULTS

Plasma membrane targeting of β -catenin activates TCF/LEF-dependent transcription and circumvents the requirement of the C-terminal transcriptional activation domain

To determine whether membrane-targeted forms of β -catenin can activate Wnt signalling, the CK1 phosphorylation site in β -catenin, Ser-45, was mutated to alanine, rendering the protein stable. The full length coding sequence of S45A mutant β -catenin was then fused in-frame to the C-terminus of the ErbB2 receptor in which the tyrosine kinase domain was deleted (Erb-cat[S45A]). Whereas non-targeted stable β -catenin (cat[S45A]) showed diffuse distribution in both the nucleus and

Figure 1 Activation of TCF/LEF-dependent transcription by plasma membrane-tethered S45A β -catenin

(a) HEK-293 cells were transfected with cat[S45A] or Erb-cat[S45A] for 2 days and immunostained with V5-antibody. (b, c) Multiple wells of HEK-293 cells were co-transfected with equal amounts of Topflash DNA and empty vector, cat[wt], cat[S45A] or Erb-cat[S45A] for 2 days and Topflash activity (b) and protein expression (c) were measured as described in the Experimental section. (d, e) Cells were co-transfected with Topflash DNA, Erb-cat[S45A] and dnTCF as indicated, and Topflash activity (d) and protein expression of Erb-cat[S45A] (e) were measured as described in the Experimental section.

cytoplasm when transfected into HEK-293 cells, Erb-cat[S45A] was exclusively localized to the plasma membrane (Figure 1a). The TCF/LEF- β -catenin-dependent reporter activity (Topflash) was then utilized to measure the ability of the different constructs to activate Wnt signalling. As shown in Figures 1(b) and 1(c), transfection of non-targeted wild-type β -catenin (cat[wt]) resulted in barely detectable protein levels and increased the reporter activity by 4-fold. Non-targeted S45A mutant β -catenin activated Wnt signalling to a much greater degree (51-fold; Figure 1b), consistent with a significantly enhanced protein expression (Figure 1c). Membrane-targeted S45A mutant β -catenin was even more potent in activating Wnt signalling (96-fold; Figure 1b), and this effect of Erb-cat[S45A] was blocked when dominant-negative TCF4 (dnTCF) was co-expressed (Figure 1d).

We next determined whether the C-terminal transactivation domain of β -catenin is required for the membrane-tethered β -catenin to activate Wnt signalling. Deletion of the C-terminus after the last Arm repeat (residues 667–781) from membrane-tethered and non-targeted β -catenin did not affect the subcellular localization (results not shown) or protein expression

levels (Figure 2a). As shown in Figure 2(b), C-terminally truncated, membrane-tethered S45A- β -catenin (Erb-cat[S45A] Δ CT) retained the ability to activate Topflash activity, albeit to a somewhat lesser extent. In contrast, deletion of the C-terminus in non-targeted S45A- β -catenin (cat[S45A] Δ CT) abrogated transcriptional activity almost completely. Addition of a farnesylation sequence to cat[S45A] Δ CT (cat[S45A] Δ CT-FAR) resulted in membrane localization (Figure 2c) and a 14-fold increase in reporter activity compared with control cells (Figure 2d).

Activation of TCF/LEF-dependent transcription by membrane-tethered β -catenin is not dependent on competition with endogenous β -catenin for binding to E-cadherin

To test whether the effect of membrane-tethered β -catenin depends on its interaction with E-cadherin at the plasma membrane by competing for binding with endogenous β -catenin, we generated a membrane-tethered form of β -catenin, in which the

Figure 2 Transcriptional activity of C-terminal deletion mutants

(a, b, d) HEK-293 cells were transfected with constructs as indicated and Topflash activity and protein expression from total lysates were measured as described under the Experimental section. (c) HEK-293 cells were transfected with cat[S45A] Δ CT-FAR for 2 days and immunostained using V5 antiserum.

C-terminus and the last two Arm repeats were deleted (Erb-cat[S45A]R10). This deletion has previously been reported to be deficient in interacting with E-cadherin [21,22]. As shown in Figure 3, Erb-cat[S45A]R10 retained the ability to activate TCF/LEF-dependent transcription. We also generated a construct which targeted β -catenin to the cytosolic face of the outer mitochondrial membrane (cat[S45A]OMM) by attaching residues 492–520 of monoamine oxidase B to the C-terminus of non-degradable β -catenin [18]. Correct subcellular localization of the expressed protein was confirmed by staining the cells with MitoTracker Red and immunostaining with V5 antiserum (Figure 4a). The expression level of cat[S45A]OMM was low compared with non-targeted stable β -catenin (Figure 4b). Interestingly, a marked increase in expression was observed in the presence of the proteasome inhibitor MG-132, indicating that the cat[S45A]OMM protein was rapidly degraded by the 26S proteasome (Figure 4b). Despite low expression levels, β -catenin targeted to the outer mitochondrial membrane activated TCF/LEF-dependent transcription significantly (Figure 4c). Because of the complication of the rapid degradation of the cat[S45A]OMM protein, we generated a construct in

Figure 3 Activation of TCF/LEF-dependent transcription by membrane-tethered S45A β -catenin lacking the C-terminal transactivation domain and the last two Arm repeats

HEK-293 cells were transfected with Topflash DNA and empty vector, cat[S45A] or membrane-tethered S45A- β -catenin lacking the C-terminus and the last two Arm repeats (Erb-cat[S45A]R10).

which residues 211–239 of Bcl-2, encoding for the transmembrane domain [23], was fused to the C-terminus of non-degradable β -catenin. Immunostaining of cells transfected with this cat[S45A]Bcl2-TM construct showed a speckled staining pattern (results not shown), consistent with localization to the endoplasmic reticulum and outer mitochondrial membrane. The cat[S45A]Bcl2-TM protein was expressed well and activated transcription to a greater extent than non-targeted β -catenin (Figure 5). Taken together, these results indicate that the ability of membrane-targeted β -catenin to activate TCF/LEF-dependent transcription does not depend on its interaction with E-cadherin.

Stable β -catenin targeted to the nucleus is unable to activate TCF/LEF-dependent transcription

We next studied the effect of non-degradable β -catenin targeted to the nucleus by the addition of NLSs (nuclear localization sequences). Two NLSs were attached to either the N- or the C-terminus of S45A- β -catenin. However, transfection of both constructs resulted in significant residual protein staining in the cytosol (results not shown). Only when two NLSs were attached to both the N-terminus and the C-terminus (cat[S45A]nucl), complete nuclear localization was observed (Figure 6a). Transfection of the construct carrying an N- and C-terminal NLS, however, failed to activate significantly TCF/LEF-dependent transcription, in spite of a similar level of protein expression compared with non-targeted stable β -catenin (Figure 6b). Interestingly, expression of the stable β -catenin constructs carrying either the N- or C-terminal NLS, both of which show residual cytoplasmic staining, activated TCF/LEF-dependent transcription to a similar degree to levels intermediate between cat[S45A] and cat[S45A]nucl (results not shown). (We also confirmed that the presence of the C-terminal V5 epitope tag did not affect the ability of cat[S45A] with an N-terminal NLS to activate transcription.) These results indicate that the failure of the cat[S45A]nucl construct to activate transcription is not due to the presence of an NLS at the C-terminus, as has been suggested [13], and that targeting of β -catenin to the nucleus correlates negatively with activation of TCF/LEF-dependent transcription.

Figure 4 Effect of S45A β -catenin targeted to the outer mitochondrial membrane on TCF/LEF-dependent transcription

(a) HEK-293 cells were transfected with cat[S45A]OMM for 2 days, then incubated with Mitotracker Red with subsequent fixing and immunostaining of cells with V5 antiserum, as described in the Experimental section. (b) Cells were transfected with cat[S45A]OMM for 2 days and treated with 25 μ M MG-132 for the last 18 h as indicated. (c) Topflash activity and protein expression were determined after co-transfection of Topflash DNA and cat[S45A] or cat[S45A]OMM, as indicated. Total DNA concentrations were adjusted with empty pcDNA3 vector.

Activation of TCF/LEF-dependent transcription by non-degradable β -catenin targeted to different cellular compartments correlates with stabilization of cytosolic wild-type β -catenin

It has been shown previously that the expression of plasma membrane-tethered β -catenin increases soluble wild-type β -catenin levels, and it was proposed that this effect accounts for the signalling ability of membrane-targeted forms of β -catenin. We therefore determined the effect of the differently targeted non-degradable forms of β -catenin on cytosolic wild-type protein. We first generated a cell line which stably expressed Erb-cat[S45A] under the control of a tetracycline-inducible promoter. As shown in Figure 7(a), Erb-cat[S45A] protein was only expressed in the presence, but not in the absence of tetracycline. Induction of Erb-cat[S45A] protein expression resulted in marked stabilization of endogenous soluble β -catenin (Figure 7a). Similarly, when wild-type β -catenin was co-transfected with Erb-cat[S45A], stabilization of the cytosolic fraction of the wild-type protein was observed (Figure 7b). To test the effect of the cat[S45A]Bcl2-TM and cat[S45A]nucl constructs on cytosolic wild-type β -catenin, we co-transfected wild-type β -catenin carrying a C-terminal HA-

Figure 5 Activation of TCF/LEF-dependent transcription by S45A β -catenin targeted to the outer mitochondrial and endoplasmic reticulum membranes

Topflash activity and protein expression were determined after co-transfection of Topflash DNA and cat[S45A] or cat[S45A]Bcl2-TM, as indicated. Total DNA concentrations were adjusted with empty pcDNA3 vector.

Figure 6 Transcriptional activity of S45A β -catenin targeted to the nucleus

(a) Immunostaining of HEK-293 cells transfected with cat[S45A]nucl with V5 antiserum and co-staining with DAPI. (b) Topflash activity and protein expression were determined after co-transfection of Topflash DNA and cat[S45A] or cat[S45A]nucl, as indicated.

epitope tag. Transfection of cat[S45A]Bcl2-TM increased the level of cytosolic HA- β -catenin markedly (Figure 7c). In contrast, cat[S45A]nucl, despite being expressed at higher levels, had only a small effect on the cytosolic HA- β -catenin concentration.

DISCUSSION

A recent study in *Drosophila* [12], as well as previous studies (see [13] and references therein), have shown that plasma

Figure 7 Effect of S45A β -catenin targeted to different cellular compartments on cytosolic wild-type β -catenin

(a) HEK-293 cells stably expressing Erb-cat[S45A] under a tetracycline inducible promoter, were cultured in the presence or absence of 1 μ g/ml tetracycline for 12 h. Cells were then lysed in hypotonic lysis buffer (to determine endogenous wild-type cytosolic β -catenin, using β -catenin antiserum) or RIPA lysis buffer (to determine Erb-cat[S45A] expression, using V5 antiserum). (b) Cells were co-transfected with wild-type V5- β -catenin and Erb-cat[S45A] or empty vector, as indicated, and concentrations of cytosolic wild-type β -catenin were measured with V5 antiserum using hypotonic cell lysates. (c) Cells were co-transfected with wild-type HA- β -catenin and empty vector, cat[S45A]Bcl2-TM or cat[S45A]nucl, as indicated, and cytosolic wild-type β -catenin was measured with HA antiserum using hypotonic cell lysates.

membrane-targeted, non-degradable forms of Arm/ β -catenin can activate TCF/LEF-dependent transcription. In the present study, using a mammalian expression system, we have confirmed these results. The effect of membrane-tethered Arm on transcription was even greater than that of non-targeted β -catenin at comparable expression levels, despite the fact that some of the non-targeted transfected protein was localized to the nucleus. Furthermore, as shown previously [12,24], membrane targeting bypasses the requirement of the C-terminus of β -catenin, which contains the transactivation domain. The failure of C-terminally truncated non-targeted β -catenin to activate transcription may be due to a dominant-negative effect, as this mutant can bind to TCF/LEF transcription factors, but is unable to recruit essential co-activators [24]. Plasma membrane-tethered, non-degradable β -catenin markedly increased endogenous β -catenin, as observed previously [14,25]. A number of reports have provided evidence that this increase in endogenous cytosolic β -catenin probably accounts for the ability of membrane-targeted β -catenin to activate TCF/LEF-dependent transcription [14–16]. A recent study by Cong et al. [26] has added further support for such a mechanism. The authors used a number of different approaches, including reducing endogenous β -catenin concentrations using RNA interference, which led to a marked decrease in the transcriptional activity induced by membrane-tethered β -catenin or plakoglobin, a relative of β -catenin.

The increase in soluble wild-type β -catenin has been suggested to be due to competition with binding to cellular β -catenin-binding proteins, i.e. E-cadherin at the plasma membrane, Axin

or APC. To determine whether the signalling capability of membrane-tethered β -catenin is confined to it as a result of it being targeted to the plasma membrane (and thus dependent on competition with endogenous β -catenin for binding to E-cadherin), we generated constructs which directed non-degradable β -catenin to the cytosolic side of the outer mitochondrial or endoplasmic reticulum membrane. These intracellular membrane-targeted β -catenin proteins also activated transcription, and when taking into account the different expression levels, their effect was even greater compared with non-targeted, non-degradable β -catenin. Similar to plasma membrane-tethered, non-degradable β -catenin, targeting to the outer mitochondrial and endoplasmic reticulum membranes increased the concentration of wild-type cytosolic β -catenin. We also showed that a plasma membrane-tethered form of β -catenin, not capable of binding E-cadherin, still activated TCF/LEF-dependent transcription. Taken together, these findings indicate that the signalling capability of membrane-targeted β -catenin does not depend on competition with endogenous β -catenin for binding to E-cadherin. The results suggest that membrane-targeted β -catenin competes with binding of endogenous cytosolic β -catenin to Axin and/or APC and thus inhibits the degradation of endogenous β -catenin, leading to its accumulation. Indeed, in a recent study, Cliffe et al. [27] reported that expression of plasma membrane-targeted Arm leads to a dramatic relocation of Axin and APC to the membrane. Cong et al. [26] have shown that the signalling activity of membrane-tethered β -catenin, but not of non-targeted β -catenin, is compromised on mutation of Trp-383 and Arg-386 in β -catenin to alanine, which results in defective binding to APC.

We finally investigated the transcriptional activity of non-degradable β -catenin targeted to the nucleus through the addition of NLSs. There are contradictory results in the literature regarding the ability of such constructs to activate Wnt signalling [12,14,15], and it has been suggested that the observed differences may be due to the different attachments of the NLS to the N- or C-terminus of β -catenin [13]. We therefore generated constructs that carried an NLS at either the N- or the C-terminus. We found that both constructs activated TCF/LEF-dependent transcription similarly, but to a lower degree compared with non-targeted β -catenin. However, targeting of β -catenin to the nucleus by both constructs was incomplete. Only when the NLSs were attached to both the N- and the C-terminus, complete nuclear localization was observed. This construct could not induce significant transcriptional activation. The results with β -catenin proteins carrying NLSs, together with the findings that the different membrane-targeted forms of β -catenin activated transcription to a greater degree than non-targeted constructs, indicate that the transcriptional activity correlates negatively with targeting of β -catenin to the nucleus. Our findings are consistent with the study by Chan and Struhl [12]. In contrast, Cox et al. [15] and Miller and Moon [14] observed that Arm and β -catenin, carrying an NLS, had signalling activity. However, attaching a nuclear localization to Arm failed to direct the protein to the nucleus when expressed in *Drosophila* [15]. β -catenin carrying an NLS expressed in *Xenopus* was predominantly localized to the nucleus, but faint staining was also observed at the plasma membrane [14]. It is possible that the fraction of overexpressed β -catenin localized to the membrane was sufficient to lead to an increase in endogenous cytosolic β -catenin. We found that exclusive targeting of β -catenin to the nucleus failed to increase significantly the concentration of cytosolic wild-type β -catenin. Taken together, our results suggest that cytosolic accumulation is a prerequisite before β -catenin can presumably translocate into the nucleus to activate gene expression. Transfected, non-degradable

β -catenin carrying NLSs will, upon synthesis, directly translocate into the nucleus. In contrast, non-targeted β -catenin is free to engage with various binding partners in the cytosol. This may lead to an activation of β -catenin, for instance by an unidentified post-translational modification or recruitment of other cofactors which are required for the activation of gene expression in the nucleus.

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