Chapter 2

Wnt signaling controls the phosphorylation status of β-catenin

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Abstract

At the heart of the canonical Wnt signaling cascade, APC, axin and GSK3 constitute the so called destruction complex which controls the stability of β-catenin. It is generally believed that four conserved Ser/Thr residues in the N-terminus of β-catenin are the pivotal targets for the constitutively active serine kinase GSK3. In cells that do not receive Wnt signals, GSK is presumed to phosphorylate β-catenin, thus marking the latter for proteasomal degradation. Wnt signaling inhibits GSK3 activity. As a consequence, β-catenin would no longer be phosphorylated and accumulate to form nuclear complexes with TCF/LEF factors. Although mutations in or near the N-terminal Ser/Thr residues stabilize β-catenin in several types of cancer, the hypothesis that Wnt signaling controls phosphorylation of these residues remains unproven. We have generated a monoclonal antibody, which recognizes an epitope containing two of the four residues when both are not phosphorylated. The epitope is generated upon Wnt signaling as well as upon pharmacological inhibition of GSK3 by lithium, providing formal proof for the regulated phosphorylation of the Ser/Thr residues of β-catenin by Wnt signaling. Immunohistochemical analysis of mouse embryos utilizing the antibody visualizes sites that transduce Wnt signals through the canonical Wnt cascade.
Introduction

Wnt signal transduction controls multiple developmental events throughout the animal kingdom. Wnt/wingless proteins constitute a large family (> 19 members in human) of secreted cysteine-rich glycoproteins. Wnts function as ligands for a receptor complex consisting of one of several members of the Frizzled (Fz) family of serpentine receptors 1 and of LRP-5/6, homologues of the LDL receptor 2-4.

β-catenin participates in a large cytoplasmic protein complex, containing the serine/threonine protein kinase glycogen synthase kinase-3 (GSK-3), the tumor suppressor gene product APC (adenomatous polyposis coli) and axin/conductin 5-8. In the absence of Wnt, GSK-3 9;10 is constitutively active, and is believed to promote degradation of β-catenin by N-terminal phosphorylation and subsequent ubiquitination and proteasomal targeting 11;12. In response to Wnt, the activity of GSK-3 is inhibited by an incompletely understood mechanism. As a consequence, β-catenin’s breakdown is suppressed 13. Indeed, oncogenic mutations in β-catenin at putative GSK-3 phosphorylation sites stabilize β-catenin in colorectal cancer and melanoma 14, causing aberrant activation of TCF 14;15. Interspecies sequence comparison of β-catenin as well as the oncogenic point mutations in β-catenin have implied four N-terminal residues as the targets of upstream kinases: Ser33, Ser37, Thr41 and Ser45 (for an overview: see URL www.ana.ed.ac.uk/rnusse/pathway/bcatmut.html). To date however, no biochemical data exist to confirm the regulated phosphorylation of these residues. Such analyses are complicated by the fact that two separate pools of β-catenin co-exist in epithelial cells. Independent of the pool of labile, APC-bound β-catenin, a much larger, highly stable pool of β-catenin is tightly bound to the cytoplasmic tail of cadherin-type adhesion molecules. This second pool effectively links cadherins to the actin cytoskeleton 16-18.

To provide formal biochemical proof for the regulated phosphorylation of β-catenin during Wnt signaling, we have developed a monoclonal antibody, αABC (α-active β-catenin), which recognizes non-phosphorylated Ser37 and Thr41. We show that the antibody visualizes the generation of active β-catenin by the canonical Wnt pathway during murine embryogenesis.

Materials and Methods

Cell lines and reagents

The 293T cell line was cultured in RPMI, complemented with 10 % fetal calf serum, penicillin and streptomycin. ALLN (N-acetyl-Leu-Leu-norleucinal) was obtained from Sigma, LiCl from Riedel de Haen.

Generation of an α-active β-catenin (αABC) antibody

A 300 bp PCR product encoding the first 100 amino acids of human β-catenin protein was cloned into pET21a prokaryote expression vector [Novagen] and used to generate His-tagged recombinant protein in BL21 E. coli. Six week old BALB/c mice were immunized with 200 µg of purified β-catenin (1-100) fusion protein in Freund’s complete adjuvant [Difco], with a second injection in Freund’s incomplete adjuvant [Difco] 14 days later. Following sacrifice of immunized mouse, the splenocytes were fused to SP2/0 mouse myeloma cells using polyethylene glycol. The fused cell population was resuspended in hypoxanthine aminopterin thymidine selection medium [Life] and plated into 96-well flat bottom culture plates. Hybridoma supernatants were screened by immunohistochemical staining of methanol-fixed COS cells transfected with human β-catenin.

The α-catenin N-terminal deletion contracts were generated in pCDNA3neo [Invitrogen] by polymerase chain reaction (PCR) and subcloned in the pGEM-T vector [Promega]. The following primers were used:

WTmyc 5’: CCA AGG ATC CAC CAT GGA GCA GAA GCT GAT CAG CGA GGA CCT GAT GGC TAC TCA AGC TGA TTT G, Δ26 5’: CCA AGG ATC CAC CAT GGA GCA GAA GCT GAT CAG CGA GGA CCT GAT CAG CGA GGAGGA CCT
GCA ACA GTC TTA CCA CCT GGA CCT T, Δ31 5': CCA AGG ATC CAT CAT GGA GCA GAA GCT GAT CAG CGA GAGAGG CCT GGA CTC TGG AAT CCA TTC TGG T, Δ35 5': CCA AGG ATC CAT CAT GGA GCA GAA GCT GAT CAG CGA GGA GGA CCT GCA TTC TGG TGC CAC TAC CAC A, Δ39 5': CCA AGG ATC ACC ATG GAG CAG AAG CTG ATC AGC GAG GAC CTG ACT ACC ACA CCT CCT TCT CTG, Δ43 5': CCA AGG ATC CAT GGA GCA GAA GCT GAT CAG CGA GAGAGG CCT GCC TTC TCT GAG TGG TAA AGG C, Δ48 5': CCA AGG ATC CAT CAT GGA GCA GAA GCT GAT CAG CGA GGA GGA CCT GAA GAG CAA TCC TGA GGA AGA G, Δ60 5': CCA AGG ATC ACC CAT GGA GCA GAA GCT GAT CAG CGA GGA GGA CCT GCA AGT CCT GTA TGA GTG GAA A, Δ 3': CGG TGG CCT CGA GCA TCT GCC TGT CCA TC. The construction of pCIneo-S33Y-β-catenin and pCIneo-Δ5 β-catenin, encoding a β-catenin molecule with an S to Y substitution or a deletion of amino acid S45, was described previously. 14,106 Cos cells were transfected with 1 µg of the β-catenin constructs using DEAE Dextran and fixed in methanol after 2 days. After rinsing the plates, the cells were incubated with the supernatants of the hybridomas for 60 min, followed by the peroxidase labeled Rabbit-α-Mouse 1:100 [DAKO] for 30 min and AEC/H2O2. Pepsan analyses were performed by Pepscan BV., Lelystad, The Netherlands.

Peptide ELISA
Covalink ELISA plates [Nunc] were activated by incubating with 100 µl 10 mM SPDP (3-(2-pyridyldithio)propionic acid N-hydroxysuccinimide ester) [Sigma] in PBS for 30 min at 37°C. The peptides used, were S33P: CQQLSYLD S(-P) GIHSG, S37P: CLDSGIH S(-P) AGTTT and T41P: CIHSGAT T(-P) TAPSL. 10 µM peptides in 0.1 MTris-HCl, pH 8.0 were directly coupled to the bivalent linker via the N-terminal cystein residue for 60 min at 37°C. The coated plates were blocked with PBS/0.25% BSA during 15 min. The monoclonal antibodies were allowed to bind for 45 min at 37°C followed by the secondary antibody Rabbit-α-Mouse peroxidase labelled [DAKO] for 30 min at 37°C. The staining was visualized by adding ABTS (2',2'-Azino-Bis(3-Ethylbenzthiazoline-6-Sulphonic Acid) Di ammonium, Boehringer)/H2O2 and measuring the absorbance at 405 nm.

Western Blots
For detection of endogenous β-catenin protein, 106 sample cells were directly lysed in 200 µl of hot 2X SDS-PAGE sample buffer (15 mM 1 MTris pH6.8, 5% SDS, 40% Glycerol, 0.005% BPB, 8% β-MeOH), vortexed vigorously, denatured peroxidase activity was blocked by incubating the sections in 1.5% peroxide in methanol for 20 minutes. The sections were then boiled in 0.01 mol/L citrate buffer, pH 6.0, for 20 minutes and cooled slowly. Before staining, the sections were blocked with Rodent Block [Labvision] for 60 min. The sections were washed in phosphate buffered saline (PBS) and incubated with hybridoma culture supernatant of αABC or with the TL pan-β-catenin antibody [Transduction Laboratories, clone 14] followed by a horse-radish-peroxidase-conjugated rabbit anti-mouse IgG polyclonal antibody [Pierce], and finally visualized by enhanced chemiluminescence [Amersham].

Immunohistochemistry
Mouse embryos were harvested between time points E10.5 and E16.5 days, and fixed in formalin at 4°C. They were subsequently embedded in paraffin and sectioned at 5 µm thickness. After deparaffinization, endogenous peroxidase activity was blocked by incubating the sections in 1.5% peroxide in methanol for 20 minutes. The sections were then boiled in 0.01 mol/L citrate buffer, pH 6.0, for 20 minutes and cooled slowly. Before staining, the sections were blocked with Rodent Block [Labvision]. The sections were washed in phosphate buffered saline (PBS) and incubated with hybridoma culture supernatant of αABC or with the TL pan-β-catenin antibody [Transduction Laboratories Inc.] diluted 1:100 in culture medium for 60 minutes. The sections were washed in PBS and the primary antibody was detected with Poly-HRP-goat-α-mouse IgG [Immunovision] and DAB solution.

Results and Discussion

Generating the dephosphory-specific αABC antibody
To generate a monoclonal antibody against dephosphorylated β-catenin, recombinant protein consisting of the N-terminal 100 amino acids of β-catenin was produced in E. coli and used for mouse immunizations. In two fusion experiments, a total of 16 hybridomas were isolated that recognized full-length human β-catenin when overexpressed in COS cells (Fig 1A). To map the epitopes recognized by these relevant monoclonal antibodies, we made a series of myc-tagged N-terminal deletion constructs (Δ26, Δ31, Δ35, Δ39, Δ43, Δ48 and Δ62; numbered for the first amino acid of β-catenin to be expressed). These constructs were transfected into COS cells. Immunohistochemical stainings revealed that 2 hybridomas recognized epitopes coinciding with the
region harboring the regulatory Ser/Thr residues (data not shown). This region comprises the potentially phosphorylated residues Ser33, Ser37, Thr41 and Ser45. Pepscan analysis was independently performed utilizing sequentially overlapping 12-mer peptides covering aminoacid 1 to 52 of β-catenin. This allowed the precise mapping of the epitopes recognized by all 16 antibodies. The Pepscan data of the antibody 8E7, pertinent to this study, are depicted in Fig 1B. The minimal epitope of the antibody as determined by PEPScan comprised HS\text{GATTTAP} (residues 36 to 44). The epitope identity was confirmed by transfecting Cos cells with expression constructs encoding several point mutants of β-catenin. The antibody neither bound the S37A mutant, nor the T41D mutant of β-catenin. Aminoacid substitutions at position S33 or S45 had no effect on antibody binding (Fig. 2). Next, a phospho-peptide ELISA was used to evaluate the influence of individual phosphorylation of the four Ser/Thr residues on antibody recognition. Interestingly, a phosphate group at either Ser37 or Thr41 prevented the binding of this monoclonal antibody to its epitope (Fig. 1C).

![Figure 1. Characterization of the αABC epitope. A) The N-terminal sequence of β-catenin, amino acids 14 to 50 is depicted. The black boxes indicate the minimal binding epitopes of individual monoclonal antibodies named below each box, as determined by Pepscan analysis. B) The Pepscan analysis for the antibody 8E7 (renamed αABC), is given as an example. From the overlapping sequences of the bound peptides the minimal epitope of the antibody was deduced to be H36-A43 encompassing the putative GSK targets, S37 and T41. C) Phosphorylation of either S37 ("S37P") or T41 ("T41P") abrogated binding of αABC (8E7), but the epitope was restored upon dephosphorylation by CIP treatment. S33P, a phosphorylated peptide not spanning the αABC epitope, was used as a negative control.](image-url)
Upon dephosphorylation of the phosphorylated peptides with calf intestine phosphatase the binding was restored. We tentatively named the antibody “anti-active β-catenin (αABC)” and concluded that it recognizes an epitope containing Ser37 and Thr41 residues only when both are dephosphorylated.

**Figure 2.** Confirmation of the αABC epitope by immunohisto-chemical analysis of β-catenin point mutants. Cos cells were transfected with β-catenin constructs with the indicated point mutations. The pan-β-catenin TL antibody stained all mutants. The αABC epitope was destroyed by point mutants in S37 or T41.

### Wnt signaling controls the phosphorylation status of the N-terminal regulatory residues of β-catenin

We then sought to address whether Wnt signaling could generate the αABC epitope. The accumulation of β-catenin levels was provoked in four different ways in 293T cells. Wnt-1 was transiently transfected into these cells, presumably activating Wnt signaling in an autocrine as well as a paracrine fashion. As a positive control, cells were transfected with a β-catenin construct encoding a dominant-positive mutant of β-catenin (Δ45-β-catenin), i.e. lacking one of the critical regulatory residues. Two different pharmacological treatments have been described to stabilize β-catenin. LiCl is presumed to mimic Wnt-signaling by the inhibition of GSK3 activity. LiCl is predicted to lead to the accumulation of non-phosphorylated, non-ubiquitinated β-catenin. The proteasomal inhibitor peptide ALLN is predicted to induce the accumulation of phosphorylated, ubiquitinated β-catenin. 293T cells were treated for 6 hrs with these inhibitors. All samples were analyzed by Western blotting, stained with either the C-terminal pan-β-catenin antibody (TL) or with αABC. The pan-β-catenin antibody showed an increase in β-catenin levels under all conditions (Fig. 3, top panel). Notably, treatment of the cells with ALLN led to an increase in overall β-catenin levels, due to the appearance of higher molecular weight, ubiquitinated forms of β-catenin. The αABC antibody showed similarly increased levels of dephosphorylated β-catenin upon addition of Wnt-1, Δ45-β-catenin and LiCl. However, no increase of the αABC signal was observed upon addition of ALLN (Fig. 3, bottom panel). This indicated that the increased signal obtained with the other stimuli was not merely a result of quantitative affinity differences. Rather, the αABC antibody unequivocally visualized the presence of β-catenin that is not modified at the regulatory residues S37 and T41. Moreover, the ubiquitinated bands that appeared upon ALLN treatment did not stain with the αABC antibody. This proved the notion that phosphorylation of β-catenin precedes ubiquitination.
Wnts control α-catenin phosphorylation

Figure 3. Wnt signaling generates the dephosphorylated αABC epitope. 293T cells were transfected with the indicated cDNAs, or treated with pharmacological inhibitors. Subsequently the cells were analyzed by Western Blotting with either the TL (top panel) or the αABC antibody (bottom panel). **Mock**: mock transfected. **Δ45**: transfected with the activated β-catenin Δ45 mutant. **LiCl**: treatment with 20 mM LiCl for 6 hours. **Wnt-1**: cells were transfected with Wnt-1 cDNA and cultured for 24 hours. **ALLN**: cells treated with the proteasome inhibitor ALLN for 6 hours at 1μM. All four treatments moderately increased overall levels of β-catenin as demonstrated with the TL antibody (top panel). Notably ALLN treatment induced the appearance of higher molecular weight, polyubiquitinated forms. Bottom panel: analysis with the αABC antibody revealed strong induction of dephosphorylated β-catenin upon transfection with Δ45 or Wnt-1 and LiCl treatment. ALLN treatment led to a very moderate increase in dephosphorylated β-catenin, if at all. Importantly the polyubiquitinated forms of β-catenin did not stain with αABC.

To confirm that the N-terminal modification of β-catenin indeed represented a phosphorylation event, we performed the following experiment. 293T cells were treated with LiCl or ALLN for 6 hrs, after which the samples were split. Half of the samples were incubated with calf intestine phosphatase. Subsequently, Western blotting was performed with the TL or αABC antibody. Significantly, the signal detected by the αABC antibody, but not the TL antibody, increased in the untreated control sample upon dephosphorylation with CIP (Fig. 4 lanes 1 and 4). In addition, the ALLN treated cells not only showed a specific increase of αABC signal upon dephosphorylation, but a higher, presumably ubiquitinated band appeared upon CIP treatment (lanes 3 and 6). The αABC signal from LiCl-treated samples were much less affected by CIP treatment, in concordance with the notion that LiCl should itself lead to an accumulation of non-phosphorylated β-catenin (lanes 2 and 5). Taken together, these experiments provide formal proof that Wnt signaling induces the accumulation of β-catenin, not phosphorylated at the N-terminal regulatory residues S37 and T41.

Figure 4. Dephosphorylation of cellular α-catenin generates the αABC epitope. Lysates of 293T cells, either mock treated or treated with LiCl or ALLN, were dephosphorylated with CIP (+CIP) or not further modified (-CIP). Top panel: no changes in total β-catenin levels are observed upon CIP treatment after analysis with the TL antibody. Bottom panel: CIP treatment of the lysates enhanced the expression of the αABC epitope in the untreated or ALLN treated cells (arrow heads). Notably dephosphorylation led to the generation of the αABC epitope in polyubiquitinated β-catenin (filled arrow head). Effects on the LiCl treated cells were marginal, presumably because β-catenin pool remains largely unphosphorylated upon LiCl treatment.
Visualization of activated β-catenin during embryonic development

To further demonstrate the physiological function of β-catenin phosphorylation we examined the generation of S37/T41-dephosphorylated β-catenin at defined sites during murine embryonic development. Our insights into the role of the canonical Wnt cascade during mammalian development stem largely from phenotypic analyses of transgenic/knockout mouse models in which expression of various components of the cascade is manipulated. The canonical Wnt cascade activated by Wnt/Fz interactions involves the destruction complex, β-catenin and TCF. However, Wnt/Fz interactions can activate other parallel signaling pathways. In insects, Wnt signaling activates the planar polarity pathway which deviates downstream of Dishevelled to a MAP kinase cascade 20. No vertebrate counterpart of this pathway has been described as yet. Moon and colleagues have described a vertebrate Wnt/Fz-activated pathway that involves protein kinase C and Ca\(^{++}\) mobilization 21;22. Genetic analyses of Wnt and Fz genes by gene disruption have generally yielded dramatic phenotypic abnormalities, yet do not reveal which downstream pathway is involved. Genetic manipulations of genes encoding downstream components of the canonical pathway, such as the TCFs, do provide such insights.

Previous studies in Tcf4\(^{-/-}\) mice have indicated that the formation of the crypt progenitor compartment in the small intestine is fully dependent on the presence of Tcf-4. While the transition of intestinal endoderm into epithelium proceeds normally in the absence of Tcf-4, no proliferative compartments are maintained in the prospective crypt regions of the small intestine 23. Thus, an epithelium results which is completely composed of differentiated, non-proliferating enterocytes and goblet cells. This phenotype indicates that a Wnt signal, most likely emanating from sub-epithelial stromal cells, activates a canonical Wnt cascade in crypt epithelial cells, culminating in the activation of TCF target genes by β-catenin/Tcf4 complexes.

Since the Tcf-4 phenotype first becomes visible at E16.5, we analyzed sections of E16.5 embryos by αABC staining, and compared these to sections stained with the pan-β-catenin antibody (Fig. 5A). While the latter antibody prominently stained the adhesion junctions of villus and crypt regions alike (left panel), αABC staining was weaker and restricted to the cytoplasm and nuclei of cells in the prospective crypts (right panel). This indicated that the canonical Wnt pathway was activated specifically in the prospective crypt cells as predicted, and validated αABC staining as a tool to visualize activity of the canonical Wnt pathway. Gene disruption of another TCF family member, Lef-1, affects the late embryonic development of several structures including teeth, whiskers, hair, and mammary glands 24. In accordance with the activity of the canonical Wnt pathway in these structures, active β-catenin could readily be detected in the ectodermal parts of the prospective toothbuds on day 12.5 after gestation (Fig. 5B). The same held for the follicles of the whiskers (from day 13.5 onward, Fig. 5C) and the ectodermal sites of mammary gland formation (day 16.5, Fig. 5D).
In this study, we provide formal evidence for the notion that, in the absence of Wnt signaling, the N-terminus of β-catenin is phosphorylated prior to its ubiquitination and degradation. Furthermore, we show that Wnt signaling as well as the inhibition of GSK3 lead to the accumulation of dephosphorylated β-catenin. We specifically demonstrate these phosphorylation events with the use of a novel monoclonal antibody, which recognizes an epitope containing two of the four proposed regulatory residues in β-catenin when both are non-phosphorylated. Furthermore, we demonstrate that the antibody visualizes sites that transduce Wnt signals through the canonical Wnt cascade during embryogenesis. Since Wnt/Fz interactions have been demonstrated to activate several different signal transduction cascades, the antibody may be instrumental in outlining those signaling events that activate the canonical pathway.

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Reference List


Chapter 2


