

# Regulation of S33/S37 phosphorylated $\beta$ -catenin in normal and transformed cells

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## Summary

A novel phosphorylation-specific antibody ( $\alpha$ p $\beta$ -catenin) was generated against a peptide corresponding to amino acids 33-45 of human  $\beta$ -catenin, which contained phosphorylated serines at positions 33 and 37. This antibody is specific to phosphorylated  $\beta$ -catenin and reacts neither with the non-phosphorylated protein nor with phosphorylated or non-phosphorylated plakoglobin. It weakly interacts with S33Y  $\beta$ -catenin but not with the S37A mutant. p $\beta$ -catenin is hardly detectable in normal cultured cells and accumulates (up to 55% of total  $\beta$ -catenin) upon overexpression of the protein or after blocking its degradation by the proteasome. Inhibition of both GSK-3 $\beta$  and the proteasome resulted in a rapid ( $t_{1/2}$ =10 minutes) and reversible reduction in p $\beta$ -catenin levels, suggesting that the protein can undergo dephosphorylation in live cells, at a rate comparable to its phosphorylation by GSK-3 $\beta$ . p $\beta$ -catenin interacts with LEF-1, but fails to

form a ternary complex with DNA, suggesting that it is transcriptionally inactive. Immunofluorescence microscopy indicated that p $\beta$ -catenin accumulates in the nuclei of MDCK and BCAP cells when overexpressed and is transiently associated with adherens junctions shortly after their formation. p $\beta$ -catenin only weakly interacts with co-transfected N-cadherin, although it forms a complex with the ubiquitin ligase component  $\beta$ -TrCP. SW480 colon cancer cells that express a truncated APC, at position 1338, contain high levels of p $\beta$ -catenin, whereas HT29 cells, expressing APC truncated at position 1555, accumulate non-phosphorylated  $\beta$ -catenin, suggesting that the 1338-1555 amino acid region of APC is involved in the differential regulation of the dephosphorylation and degradation of p $\beta$ -catenin.

Key words:  $\beta$ -catenin, GSK-3 $\beta$ , APC

## Introduction

$\beta$ -catenin plays a central and direct role in cell adhesion, bridging between cadherins and the actin cytoskeleton, and in the regulation of gene expression via the Wnt signaling pathway. These distinct activities are regulated through its differential interaction with several molecular partners, including cell-cell adherens junctions (AJ) molecules, components of its degradation complex, such as the tumor suppressor adenomatous polyposis coli (APC), and transcription factors of the LEF/TCF family (Behrens et al., 1996; Huber et al., 1996; Molenaar et al., 1996; Zhurinsky et al., 2000b). In AJ,  $\beta$ -catenin functions to directly link cadherins to the actin cytoskeleton via  $\alpha$ -catenin (Ben-Ze'ev and Geiger, 1998). Cytoplasmic  $\beta$ -catenin, which is not membrane-bound, forms a complex with APC, axin/conductin and glycogen synthase kinase-3 $\beta$  (GSK-3 $\beta$ ) (Kikuchi, 2000). GSK-3 $\beta$  phosphorylates  $\beta$ -catenin as well as other components of this complex. p $\beta$ -catenin is recognized by  $\beta$ -TrCP, an F-box component of the E3 ubiquitin ligase complex that recruits an E2 ubiquitin conjugating enzyme and promotes ubiquitination of  $\beta$ -catenin (Hart et al., 1999; Kitagawa et al., 1999; Sadot et al., 2000; Winston et al., 1999). The ubiquitinated protein is then targeted for degradation by the 26S proteasome system (Aberle et al., 1997). Activation of Wnt signaling involves the

inhibition of GSK-3 $\beta$  through mechanisms that may involve axin binding to the proteins Dishevelled (Li et al., 1999) or LRP-5 (Mao et al., 2001). This results in the accumulation of  $\beta$ -catenin in the nucleus, its binding to LEF/TCF transcription factors and the expression of target genes including c-myc and cyclin D1 (He et al., 1998; Shuttman et al., 1999; Tetsu and McCormick, 1999).

Wnt signaling is essential for proper embryonal development. In *Xenopus* embryos, ectopic expression of Wnt can induce secondary axis formation (Sokol, 1999). In the fruit fly, the homologous wingless pathway is involved in the establishment of segment polarity, wing formation and differentiation of the endoderm (Cadigan and Nusse, 1997). In mice, the targeting of different wnt isoforms leads to different phenotypes; Lack of Wnt 1 results in the deletion of part of the midbrain (McMahon and Bradley, 1990), the ablation of Wnt-4 affects the kidney (Stark et al., 1994), Wnt 7a affects limb development (Parr and McMahon, 1995) and *wnt3* knockout mice are deficient in the formation of the anterior-posterior axis (Liu et al., 1999).

In adult tissues, components of the Wnt signaling pathway, such as  $\beta$ -catenin and APC, regulate cell proliferation in epithelial cells lining the colon (Polakis, 1999; Polakis, 2000). Mutations that perturb the function of the  $\beta$ -catenin

degradation complex, such as truncation of APC or mutations in the GSK-3 $\beta$  phosphorylation sites of  $\beta$ -catenin, are present in 90% of colon cancers and in other types of tumors (Polakis, 2000). Although phosphorylation of  $\beta$ -catenin by GSK-3 $\beta$  plays a pivotal role in regulating the fate and activity of  $\beta$ -catenin, the properties of the phosphorylated intermediate of  $\beta$ -catenin have not been directly characterized yet. In this study we used a novel antibody that specifically recognizes S33/S37 phosphorylated  $\beta$ -catenin to study the kinetics of its phosphorylation, dephosphorylation and ubiquitination in normal and colon cancer cells, and we determined its subcellular distribution. We show that normal cells contain very low levels of p $\beta$ -catenin (<1-2% of the protein), but the levels of p $\beta$ -catenin increase upon overexpression of the protein or after blocking proteasomal degradation. p $\beta$ -catenin can be dephosphorylated by an as yet unknown phosphatase at a rate comparable to its GSK-3 $\beta$ -mediated phosphorylation in cells overexpressing  $\beta$ -catenin. p $\beta$ -catenin accumulates mainly in the nucleus but fails to form a ternary complex with LEF-1 and DNA and is not associated with AJ, except shortly after junction formation. We further show that colon cancer cells expressing different APC mutants differ in their phosphorylated  $\beta$ -catenin levels, suggesting that APC, truncated at position 1338 (as in SW480 cells), can still support phosphorylation but fails to promote degradation, whereas truncation of APC at position 1555 (as in HT29 cells) allows limited phosphorylation and degradation of  $\beta$ -catenin.

## Materials and Methods

### Preparation of anti-phosphorylated- $\beta$ -catenin antibody

A mouse monoclonal antibody was raised against a peptide containing 16 amino acids that corresponded to the  $\beta$ -catenin phosphorylation site by GSK-3 $\beta$ , which included phosphorylated serines at positions 33 and 37. Specifically, the peptide consisted of amino acids 33-45 of human  $\beta$ -catenin, flanked by N-terminal alanine and C-terminal glycine and cysteine. For immunization, the peptide was conjugated to keyhole limpet hemocyanin (KLH) using glutaraldehyde (Yao et al., 2000). BALB/c mice were immunized and their spleen cells fused with NS-1 mouse myeloma cells. Hybridoma supernatants were screened for specific antibodies by enzyme-linked immunosorbent assay (ELISA) in a 96-well plate coated with bovine serum albumin (BSA) conjugated to the double phosphorylated peptide (2  $\mu$ g/ml). After incubation with the antibody (1 hour), the wells were washed, incubated with peroxidase-conjugated secondary antibody (1 hour) and processed according to the manufacturer's instructions (Pierce, Rockford IL). For competitive ELISA, the antibody binding to the peptide-BSA conjugate was competed with 12.5, 25, 50 or 100  $\mu$ g/ml (1 hour) of non-conjugated, doubly phosphorylated, non-phosphorylated or single phosphorylated peptides on serines 33 or 37. In this study, we used the ascites fluid of a clone (BC21) that is now commercially available (# C4231 Sigma Israel).

### Cells and transfections

Bovine endothelial cells (BCAP), MDCK, 293T, Rat1 and Rat1 ras and the human colon carcinoma cell lines SW480, HCT116 and HT29 were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (FCS). For transfection, cells were plated to form a 50%-70% confluent culture in 30 mm dishes. MDCK cells were transfected using lipofectamine (GIBCO BRL, Rockville USA), and 293T cells were transfected by the calcium phosphate method. Treatments with 30 mM LiCl or 25  $\mu$ M MG132 were for the time periods indicated.

### Plasmids

Plasmids expressing HA- $\beta$ -catenin, HA-S33Y  $\beta$ -catenin, VSV- $\beta$ -catenin and GFP- $\beta$ -catenin were as described previously (Shtutman et al., 1999; Simcha et al., 1998; Zhurinsky et al., 2000b). HA- $\beta$ -catenin S37A was a kind gift from S. Byers (Orford et al., 1999). The chicken N-cadherin and  $\Delta$ F- $\beta$ -TrCP expressing plasmids were previously described (Sadot et al., 1998; Sadot et al., 2000).

### Protein analysis

Protein levels were monitored by western blotting. The following antibodies were used: monoclonal anti-phosphorylated- $\beta$ -catenin and polyclonal anti- $\beta$ -catenin were from Sigma (Rehovot, Israel); monoclonal anti- $\beta$ -catenin (clone 14c19220) was from Transduction Laboratories (Lexington, KY). A monoclonal anti-HA antibody (clone 12CA5) was from Roche (Germany) and polyclonal anti-HA (Y11 sc-805) was from Santa Cruz Biotechnology (Santa Cruz, CA). Western blots were developed using the ECL method (Amersham UK). Autoradiograms were scanned by a GS-700 imaging densitometer (Bio Rad Laboratories, Hercules, CA) using the FotoLook PS 2.07.2 software. The intensity of the bands was quantified using the NIH image 1.61 software.

### Immunofluorescence microscopy

Cells were cultured on glass coverslips, fixed with 3% paraformaldehyde in phosphate-buffered saline (PBS) and permeabilized with 0.5% Triton X-100. The coverslips were incubated with the primary antibodies as described above. The secondary antibodies were Alexa-488-conjugated goat anti-mouse or anti-rabbit IgG (Molecular Probes, Eugene, OR) and Cy3-conjugated goat anti-mouse or anti-rabbit IgG (Jackson ImmunoResearch Laboratories West Grove PA). Images were acquired using the DeltaVision system (Applied Precision, Issaquah, WA) equipped with a Zeiss Axiovert 100 microscope (Oberkochen Germany) and Photometrics 300 series scientific-grade cooled CCD camera (Tucson, AZ), reading 12 bit images, and using a  $\times$ 100/1.3 NA plan-Neofluar objective. For quantitative image processing, the Prism software was employed (Kam et al., 1993). Ratio imaging analysis was done as described previously (Zamir et al., 1999).

### Nuclear extracts and DNA mobility gel shift analysis

293T cells grown in 90 mm diameter dishes were transfected with 2  $\mu$ g of LEF-1 and 8  $\mu$ g of  $\beta$ -catenin expression plasmids or with the control pCIneo vector. 36 hours after transfection, nuclear extracts were prepared as previously described (Shtutman et al., 1999). Briefly, cells were incubated for 15 minutes in low-salt buffer, then NP-40 was added, nuclei were pelleted by centrifugation, and nuclear proteins were extracted with high-salt buffer at 4°C. Protein concentrations were determined using the bicinchoninic acid protein assay reagent (Pierce) and bovine serum albumin was used as a standard. For DNA binding assays, 6  $\mu$ g of nuclear extracts were used. 1  $\mu$ g of antibody was added to the binding reactions for analyzing the DNA mobility supershift.

## Results

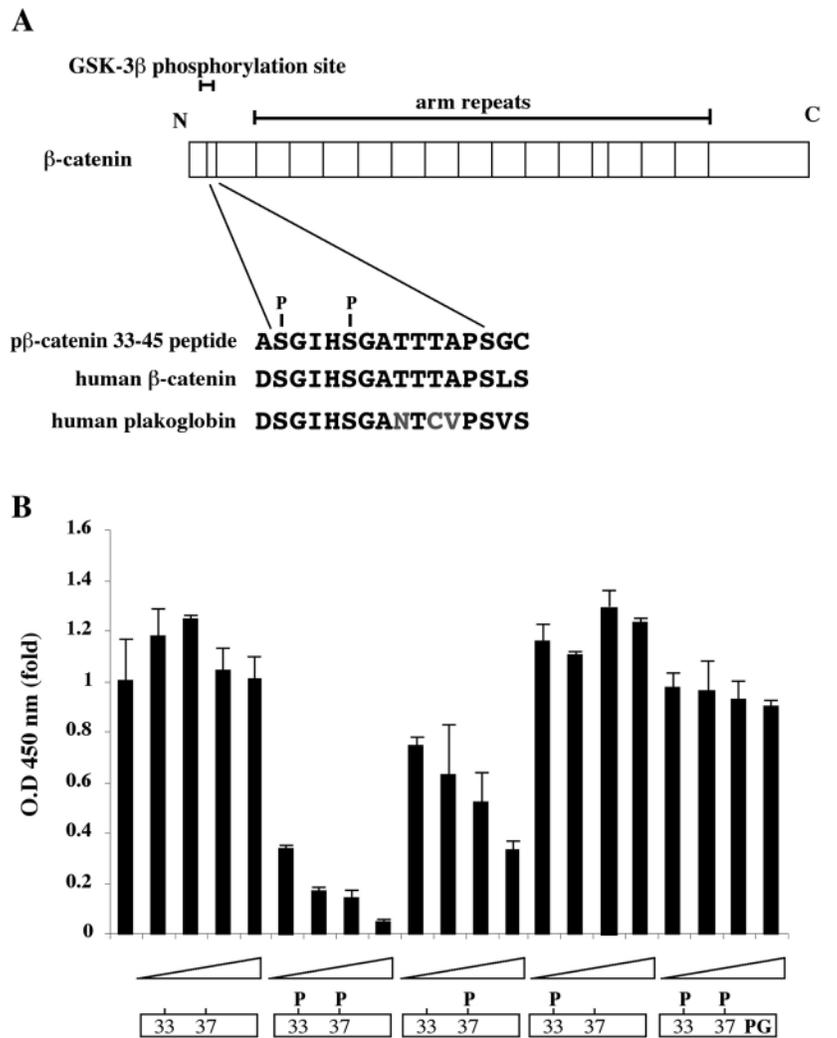
### Specificity of anti-phosphorylated- $\beta$ -catenin antibody

A novel antibody ( $\alpha$ p $\beta$ -catenin) was raised against a peptide corresponding to amino acids 33-45 of  $\beta$ -catenin; this peptide contained two phosphorylated serines, S33 and S37 (Fig. 1A, lower panel). The specificity of the  $\alpha$ p $\beta$ -catenin antibody was verified by an ELISA assay with plates coated with the double phosphorylated peptide to determine the binding of the  $\alpha$ p $\beta$ -catenin antibody in the presence of increasing amounts (0, 12.5,

25, 50 or 100  $\mu\text{g/ml}$ ) of either a non-phosphorylated, a doubly phosphorylated (S33 and S37) or singly phosphorylated (S33 or S37) peptides. As shown in Fig. 1B, the non-phosphorylated peptide did not inhibit binding of  $\alpha\beta$ -catenin to the coated plate, whereas the doubly phosphorylated peptide competed very strongly (>50% inhibition with 12.5  $\mu\text{g/ml}$  of the competitor). The singly phosphorylated peptide at S33 did not bind to the antibody, whereas the singly phosphorylated peptide on S37 inhibited weakly (50% inhibition with 100  $\mu\text{g/ml}$  of this competitor). Furthermore, a doubly phosphorylated peptide derived from plakoglobin (Fig. 1A), a close homologue of  $\beta$ -catenin, was not inhibitory (Fig. 1B, PG). These results indicate that the  $\alpha\beta$ -catenin antibody specifically and preferentially recognizes the doubly phosphorylated peptide, and there is a greater contribution of the phosphorylated S37 to the epitope.

To determine the specific interaction of the antibody with full-length  $\beta$ -catenin, 293T cells were transfected with wild-type  $\beta$ -catenin or with the two corresponding  $\beta$ -catenin mutants, S33Y (Simcha et al., 1998) and S37A (Orford et al., 1999), which were tagged with HA. Although the expression of the wild-type and mutant  $\beta$ -catenins were comparable (Fig. 2A, bottom panel), wild-type  $\beta$ -catenin was recognized by the  $\alpha\beta$ -catenin antibody, whereas the S33Y mutant reacted only weakly, and the S37A mutant was not recognized at all (Fig. 2A, upper panel). To test what fraction of  $\beta$ -catenin is phosphorylated, 293T cells were transfected with  $\beta$ -catenin together with HA-tagged  $\Delta\text{F-}\beta\text{-TrCP}$  that can bind to phosphorylated  $\beta$ -catenin (Hart et al., 1999; Sadot et al., 2000) but is unable to promote its ubiquitination and degradation.  $p\beta$ -catenin was then immunoprecipitated from the transfected cells as a complex with  $\Delta\text{F-}\beta\text{-TrCP}$  using anti-HA antibodies and immunoblotted with either the  $\alpha\beta$ -catenin antibody or with a general antibody against  $\beta$ -catenin. Comparison of the labeling intensities with the two antibodies was used for calibration (Fig. 2B). These experiments indicated that in  $\beta$ -catenin-overexpressing 293T cells, 35–55% of the transfected  $\beta$ -catenin molecules were phosphorylated (Fig. 2B).

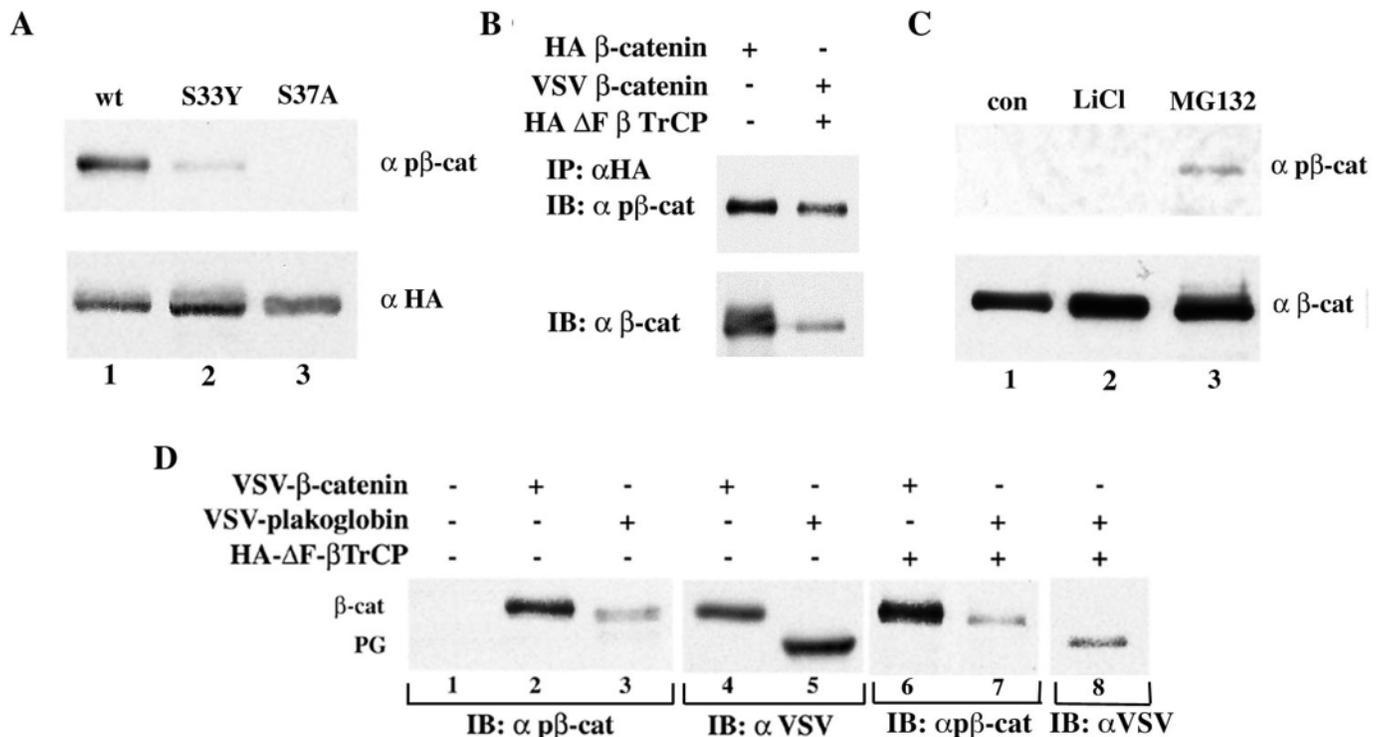
The endogenous  $\beta$ -catenin of 293T cells was not recognized by the  $\alpha\beta$ -catenin antibody, but treatment with the proteasomal inhibitor MG132 resulted in a dramatic increase in  $p\beta$ -catenin levels (Fig. 2C, lane 3 upper panel) and in only a two-fold increase in the levels of total  $\beta$ -catenin (Fig. 2C, lane 3 lower panel). Treatment with LiCl (which inhibits GSK-3 $\beta$  activity) induced an accumulation of total  $\beta$ -catenin (comparable to that found in MG132-treated cells), yet the accumulated protein was not phosphorylated (Fig. 2C, lane 2 upper panel). Taken together these results suggest that the  $\alpha\beta$ -catenin antibody is specific to the S33/S37 phosphorylated  $\beta$ -catenin and that transfected  $\beta$ -catenin accumulates as a



**Fig. 1.** Preparation and characterization of the specific anti-phosphorylated- $\beta$ -catenin antibody. (A) A schematic representation of the  $\beta$ -catenin molecule and the doubly phosphorylated peptide corresponding to its N-terminus that was used to prepare the antibody. Note the homology between  $\beta$ -catenin and plakoglobin in this region. (B) An ELISA assay for determining the specificity of the antibody. Binding of the antibody to the doubly phosphorylated peptide was detected by a peroxidase-based reaction in the absence of a competitor (first lane on the left) or in the presence of increasing amounts (12.5 25, 50 and 100  $\mu\text{g/ml}$ ) of the indicated peptide competitors.

phosphorylated molecule (Fig. 2A, lane 1). In non-transfected cells,  $p\beta$ -catenin is a short lived molecule (Fig. 2C, lane 1, compare with Fig. 2A, lane 1 upper panel and see below).

Interestingly, the  $\alpha\beta$ -catenin antibody did not recognize transfected plakoglobin in 293T cells (Fig. 2D, compare lane 3 with 5) despite the high sequence homology with  $\beta$ -catenin in the GSK-3 $\beta$  phosphorylation domain (Fig. 1A). To rule out the possibility that  $\alpha\beta$ -catenin antibody does not recognize transfected plakoglobin because it is not phosphorylated, we co-immunoprecipitated the phosphorylated form of plakoglobin with  $\Delta\text{F-}\beta\text{-TrCP}$ . As shown in Fig. 2D (lanes 7 and 8),  $\Delta\text{F-}\beta\text{-TrCP}$ -bound plakoglobin (which is most probably phosphorylated on the corresponding serine residues) is not recognized by the  $\alpha\beta$ -catenin antibody. It is worth noting that there is an increase in endogenous  $p\beta$ -catenin in the presence



**Fig. 2.** The anti p $\beta$ -catenin antibody recognizes wild-type  $\beta$ -catenin but not mutant  $\beta$ -catenin or plakoglobin. (A) HA-tagged wild-type  $\beta$ -catenin or mutant S33Y and S37A- $\beta$ -catenins were transfected into 293T cells and analyzed by western blotting with either anti-HA or  $\alpha$ p $\beta$ -catenin antibodies. (B) 293T cells were transfected with HA- $\beta$ -catenin or with VSV- $\beta$ -catenin and HA- $\Delta$ F- $\beta$ -TrCP. Immunoprecipitation was performed with an anti-HA antibody and western blotting with  $\alpha$ p $\beta$ -catenin or general  $\alpha$  $\beta$ -catenin antibodies. (C) 293T cells were treated with 30 mM LiCl overnight or with 25  $\mu$ M MG132 for 4 hours and analyzed by western blotting for p $\beta$ -catenin and total  $\beta$ -catenin content. (D) VSV-tagged plakoglobin or  $\beta$ -catenin were transfected into 293T cells, and their reactivity with  $\alpha$ p $\beta$ -catenin antibody was determined when transfected alone or together with HA-tagged  $\Delta$ F- $\beta$ -TrCP. IP, immunoprecipitation; IB, immunoblot.

of excess plakoglobin (Fig. 2D, compare lane 3 with lane 1). This is in agreement with previous reports showing that overexpression of plakoglobin attenuates the degradation of  $\beta$ -catenin (Miller and Moon, 1997; Simcha et al., 1998; Zhurinsky et al., 2000a), but, as shown here, not its phosphorylation.

#### The dynamics and fate of phosphorylated $\beta$ -catenin

In the presence of Wnt signaling,  $\beta$ -catenin phosphorylation is inhibited, thus leading to a reduction in its poly-ubiquitination and proteasomal degradation (Polakis, 2000). By an alternative process,  $\beta$ -catenin could be dephosphorylated by a phosphatase. To test whether dephosphorylation of  $\beta$ -catenin takes place in live cells, GFP- $\beta$ -catenin was transfected into 293T cells, and 24 hours later, proteasomal degradation was blocked by MG132 for 1, 2 and 4 hours in the presence or absence of LiCl to block GSK-3 $\beta$  activity. After 2 hours with MG132 and LiCl, the medium was replaced with fresh medium containing only MG132. After incubation for different periods of time (from 10 to 120 minutes), the level of p $\beta$ -catenin was determined. As shown in Fig. 3A after 10 minutes of treatment with LiCl, a dramatic drop (>50%) in the content of p $\beta$ -catenin was observed (Fig. 3A, lane 5 upper panel). Since proteasomal activity was blocked by MG132 during this time and no reduction in total  $\beta$ -catenin was observed (Fig. 3A, lower panel), we propose that the reduction

in p $\beta$ -catenin results from its dephosphorylation rather than its degradation. Removal of LiCl was followed by a rapid increase in p $\beta$ -catenin levels (Fig. 3A, lanes 9-12, upper panel). To determine the phosphorylation-dephosphorylation kinetics in non-transfected cells, 293T cells were treated with MG132 for different time periods to induce accumulation of p $\beta$ -catenin (Fig. 3B, lanes 1-5). The levels of p $\beta$ -catenin increased linearly up to four-fold after 5 hours of MG132 treatment (Fig. 3B,C). Western blotting for total  $\beta$ -catenin (Fig. 3B lane 1-5 lower panel) showed an increase in the poly-ubiquitinated protein. To determine whether dephosphorylation of p $\beta$ -catenin takes place, 293T cells were first treated with MG132 for 2 hours, and then LiCl was added for different periods ranging from 10 minutes to 2 hours. The addition of LiCl resulted in a rapid decline in p $\beta$ -catenin level (a decrease of ~60% within 10 minutes, Fig. 3C) and after 30-60 minutes with LiCl essentially no p $\beta$ -catenin was detected (Fig. 3B, lanes 6-9). It is noteworthy that only a limited decrease in total  $\beta$ -catenin was detected following this treatment (Fig. 3B, lower panel). To compare the rate of  $\beta$ -catenin dephosphorylation (in the presence of LiCl) to its rate of phosphorylation, cells treated with MG132 and LiCl (Fig. 3B lane 9) were washed and further incubated, in the absence of LiCl, for 10-60 minutes. As shown in Fig. 3B (lanes 10-12), removal of LiCl resulted in the fast recovery of p $\beta$ -catenin levels at a rate similar to the decline observed after addition of LiCl (note that MG132 was present throughout this treatment

to avoid proteasomal degradation of the protein).

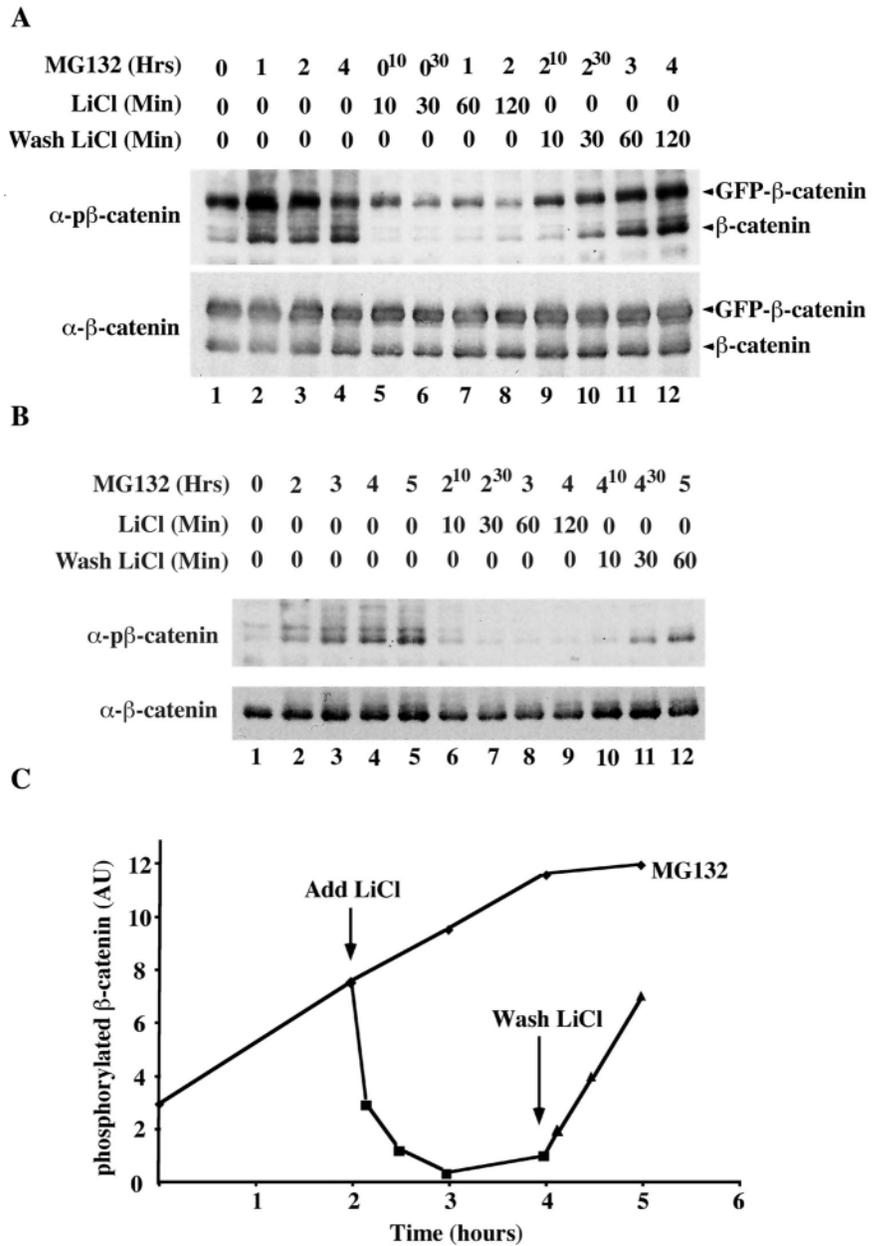
### The molecular partners of phosphorylated $\beta$ -catenin

Next, we asked whether  $p\beta$ -catenin can interact with  $\beta$ -catenin's transcriptional (LEF-1) and junctional (cadherin) partners. HA-LEF-1 and VSV- $\beta$ -catenin were co-transfected into 293T cells, immunoprecipitated with  $\alpha$ HA antibody and immunoblotted with  $\alpha p\beta$ -catenin. As shown in Fig. 4A (upper panel),  $p\beta$ -catenin could form a complex with LEF-1. We next examined whether  $p\beta$ -catenin can form a ternary complex with LEF-1 and DNA. First we verified that the  $\alpha p\beta$ -catenin antibody effectively immunoprecipitated  $p\beta$ -catenin from cell extracts in the buffer used for DNA mobility gel shift assays (Fig. 4B). Then, a gel shift analysis was performed with a radiolabelled probe corresponding to the consensus DNA sequence of the LEF/TCF-binding site incubated with nuclear extracts from 293T cells transfected with LEF-1 and  $\beta$ -catenin. As shown in Fig. 4C, although a general antibody against  $\beta$ -catenin supershifted the ternary complex of DNA-LEF-1- $\beta$ -catenin (Fig. 4C, lane 5), the  $\alpha p\beta$ -catenin antibody was unable to do so (lane 6). This suggests that  $p\beta$ -catenin is inefficient in forming a ternary complex with LEF-1 and DNA.

Interestingly,  $p\beta$ -catenin (unlike its non-phosphorylated counterpart) was also inefficient in associating with transfected N-cadherin (Fig. 5 lane 5) compared with its association with  $\Delta F$ - $\beta$ -TrCP (Fig. 5 lane 6), whereas the levels of both total and  $p\beta$ -catenin were elevated in N-cadherin-transfected cells (Fig. 5 lane 2). This suggests that  $p\beta$ -catenin is normally not associated with N-cadherin.

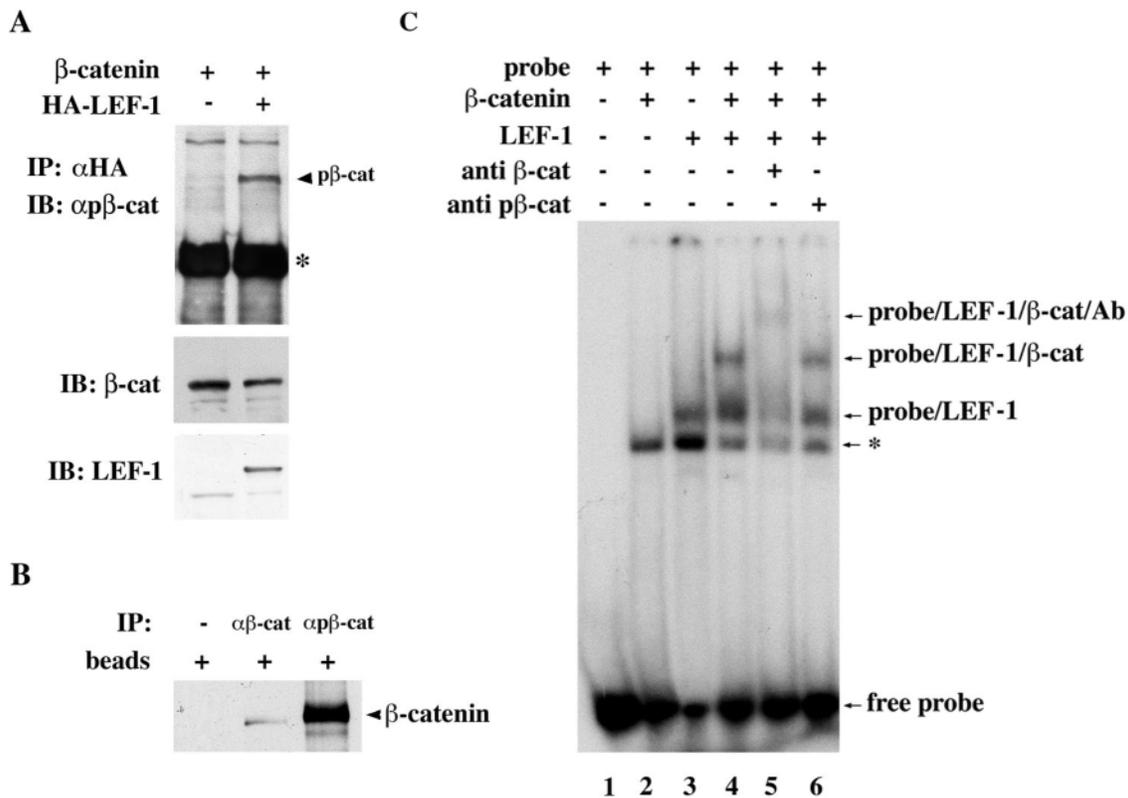
### The subcellular distribution of phosphorylated $\beta$ -catenin

Immunofluorescence labeling with  $\alpha p\beta$ -catenin from different cultured cells, including MDCKs (epithelial), BCAPs and PEACs (endothelial), as well as Rat1 and Rat1 ras (fibroblasts), was largely negative. However, the inhibition of proteasomal degradation with MG132 in BCAP cells (Fig. 6A), or after overexpression of  $\beta$ -catenin in MDCK cells (Fig. 7a), resulted in the accumulation of  $p\beta$ -catenin in the nuclei of these cells. Examination of the transfected MDCK cells using three dimensional ratio imaging (Fig. 7a) indicated that the transfected  $\beta$ -catenin (visualized using regular  $\alpha\beta$ -catenin antibody) is associated with both adherens junctions and the

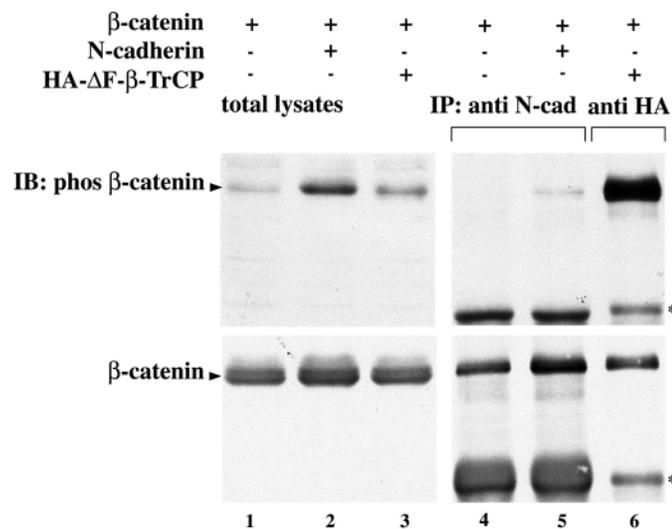


**Fig. 3.**  $\beta$ -catenin phosphorylated on serines 33 and 37 can undergo dephosphorylation. (A) GFP- $\beta$ -catenin was transfected into 293T cells, and after 24 hours the cells were treated with either MG132 (lanes 1-4), MG132 plus LiCl (lanes 5-8) or treated with MG132 and LiCl and then washed and incubated with MG132 only (lanes 9-12). (B) 293T cells were treated with MG132 (lanes 2-5), left untreated (lane 1) or incubated with MG132 and LiCl (lanes 6-9) or treated with MG132 and LiCl and then washed from the LiCl and left with only MG132 (lanes 10-12). Cell extracts in (A) and (B) were analyzed by western blotting with  $\alpha p\beta$ -catenin antibody and reprobed with general anti- $\beta$ -catenin antibody. (C) Quantitative analysis of the level of phosphorylated  $\beta$ -catenin is shown in B. The numbers correspond to intensities of the signal with  $\alpha p\beta$ -catenin antibody normalized to the signal obtained with the general anti- $\beta$ -catenin antibody.

nucleus (blue in the ratio image), whereas the  $p\beta$ -catenin is exclusively nuclear (Fig. 7a, yellow in the ratio image) and was absent from cell-cell junctions. To verify that the absence of junctional labeling for  $p\beta$ -catenin indeed resulted from the absence of the phosphorylated molecule we subjected the cells to different permeabilization procedures, none of which



**Fig. 4.** pβ-catenin can form a complex with LEF-1 but does not form a ternary complex with LEF-1 bound to DNA. (A) VSV-β-catenin was co-transfected with HA-LEF-1 into 293T cells and immunoprecipitated with anti HA antibody as a complex with LEF-1. Cell extracts were analyzed by western blotting with the αpβ-catenin antibody. The asterisk marks the Ig heavy chain of the antibody. (B) VSV-β-catenin was transfected into 293T cells and immunoprecipitated with αβ-catenin or with αpβ-catenin antibodies as indicated. (C) β-catenin-LEF-1-DNA complex formation was analyzed with 6 μg of nuclear extracts from 293T cells transfected with LEF-1 and VSV-β-catenin that were incubated with <sup>32</sup>P-labeled DNA containing the LEF-1-binding site, in the absence or presence of the indicated antibodies. Protein-DNA complexes were separated by native 4% PAGE and visualized by autoradiography. The asterisk marks a non-specific band. IP, immunoprecipitation; IB, immunoblot.

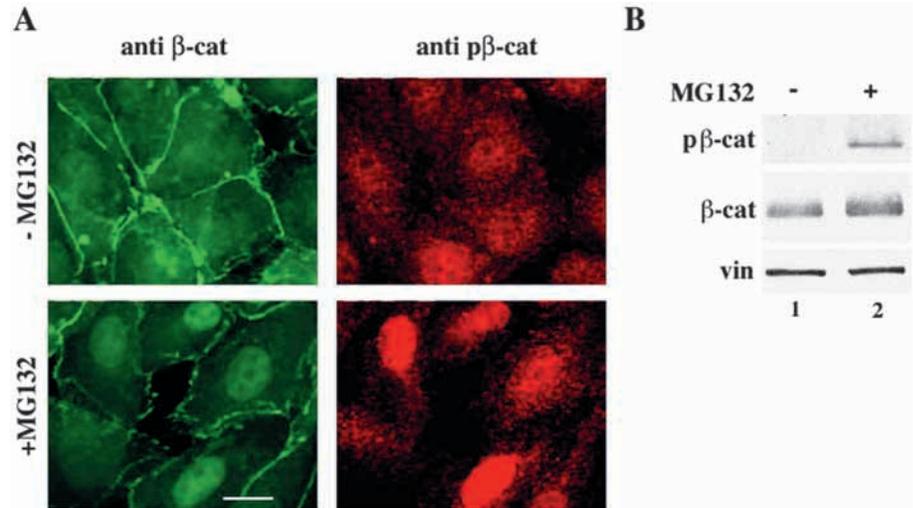


**Fig. 5.** β-catenin is hardly ever found in complex with N-cadherin. 293T cells were transfected with β-catenin together with either N-cadherin or with ΔF-β-TrCP, which served as a control for complexing with pβ-catenin. Cell extracts were immunoprecipitated as indicated and analyzed by western blotting with both the αpβ-catenin and a general anti-β-catenin antibodies. The asterisks mark the Ig heavy chain of the antibody.

revealed junctional labeling (data not shown), indicating that the lack of antibody accessibility is unlikely. Interestingly, we found that pβ-catenin is transiently localized in newly formed adherens junctions of BCAP cells (Fig. 7b). Thus, adherens junctions of BCAP cells, formed within 10 hours of plating (Fig. 7bA) or after 3 hours recovery from treatment with EGTA (Fig. 7bF) were labeled with αpβ-catenin, unlike those stained after 20-30 hours of incubation (Fig. 7bB,C). This fluorescence labeling could be readily inhibited by the synthetic, doubly phosphorylated β-catenin peptide (insert, Fig. 7bA). These results suggest that pβ-catenin can be incorporated into newly formed, but not mature, junctions and that the junction-bound phosphorylated protein is either turning over after a longer time or undergoing dephosphorylation.

#### Phosphorylation of β-catenin in colon cancer cells

The majority of colon tumors contain APC mutations, which are characterized by large deletions in the C-terminus of the protein (Polakis, 2000). This region is involved in β-catenin and axin binding, and its deletion leads to the accumulation of β-catenin. Using the novel αpβ-catenin antibody we determined the levels of pβ-catenin before and after blocking proteasomal degradation in several colon cancer cell lines. HT29 (APC truncation at position 1555), SW480 (APC



**Fig. 6.** Blocking proteasomal degradation leads to nuclear accumulation of  $\beta$ -catenin. (A) BCAP cells were treated with MG132 for 4 hours and then fixed and stained as indicated. Bar, 10  $\mu$ m. (B) Cell extracts from the MG132-treated and control BCAP cells were analyzed by western blotting. vin, vinculin; p $\beta$ -cat, phosphorylated  $\beta$ -catenin.

truncation at position 1338) and HCT116 (wild-type APC and  $\Delta$ Ser45  $\beta$ -catenin) were compared with 293T cells containing wild-type APC and  $\beta$ -catenin. The levels of  $\beta$ -catenin were found to be higher in the three colon cancer cell lines compared with 293T cells, as expected (Fig. 8, lane 1, compare lane 3 with 5 and 7). Interestingly, blocking proteasomal degradation resulted in a five-fold increase in the content of p $\beta$ -catenin in HT29 and HCT116 (Fig. 8, compare lanes 3 to 4 and 7 to 8), whereas a more than 50-fold increase was observed in 293T cells (Fig. 8, lanes 1 and 2). This suggests that the mutant APC in HT29 is at least partially capable of supporting degradation of  $\beta$ -catenin. In HCT116 cells the p $\beta$ -catenin may be derived from the wild-type molecule generated from the intact allele (Morin et al., 1997). In addition, a high content of p $\beta$ -catenin (20% of the total  $\beta$ -catenin) was observed in SW480 cells. This is in agreement with a previous observation that  $\beta$ -TrCP can bind to  $\beta$ -catenin from SW480 cells in the absence or presence of a proteasomal inhibitor (Hart et al., 1999). This suggests that the relatively high amounts of  $\beta$ -catenin accumulating in SW480 cells might saturate the  $\beta$ -catenin degradation machinery or that the region between amino acid 1338 (SW480) and 1555 (HT29) in the APC molecule may play an important role in the degradation or dephosphorylation of p $\beta$ -catenin.

## Discussion

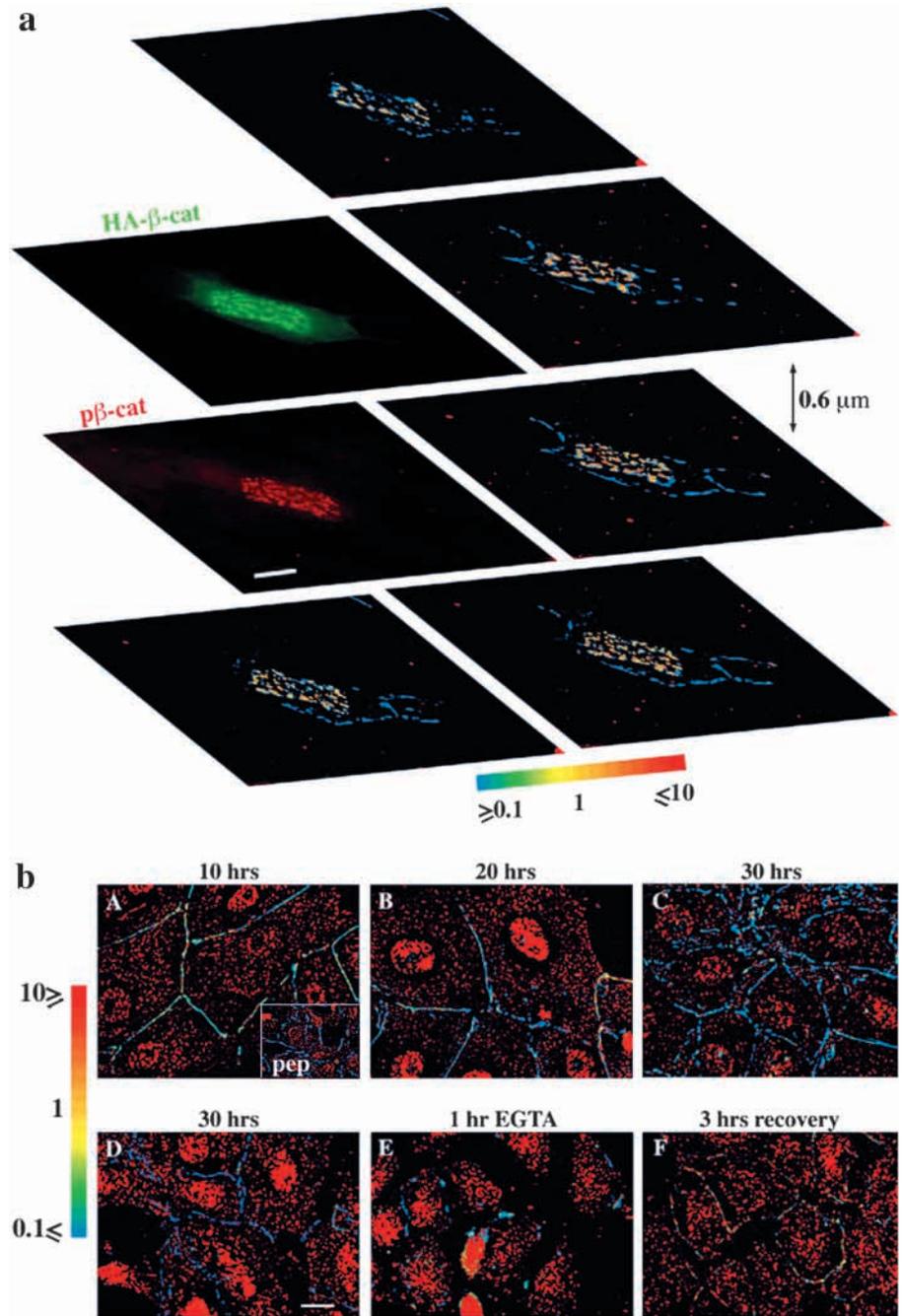
In this work we used a novel antibody that specifically recognizes  $\beta$ -catenin phosphorylated on serines 33 and 37 to follow this important intermediate in  $\beta$ -catenin degradation in cultured cells. When Wnt signaling is inactive,  $\beta$ -catenin is phosphorylated and subsequently ubiquitinated and degraded by the 26S proteasomal system. We addressed here the following questions: (i) what is the level of endogenous p $\beta$ -catenin in normal and in colon cancer cells; (ii) what are the relative rates of phosphorylation and possible dephosphorylation of the protein; (iii) does p $\beta$ -catenin interact with the 'classic' partners of  $\beta$ -catenin; and (iv) does the phosphorylation of  $\beta$ -catenin affect its subcellular organization.

An essential pre-requisite for addressing these questions was to obtain a highly specific antibody ( $\alpha$ p $\beta$ -catenin) for tracing

the phosphorylated molecule. Our study indicates that this antibody interacts primarily with the doubly phosphorylated  $\beta$ -catenin, either the endogenous or when transfected, tagged (with HA, VSV or GFP) or untagged.  $\beta$ -catenin phosphorylated at position S37 showed low reactivity with  $\alpha$ p $\beta$ -catenin, whereas  $\beta$ -catenin phosphorylated on S33 and the closely homologous protein plakoglobin were negative. It was thus concluded that  $\alpha$ p $\beta$ -catenin is highly specific for p $\beta$ -catenin on both S33 and S37.

Examination of different epithelial, endothelial and mesenchymal cell lines indicated that all, except for the colon cancer cell line SW480 (see below), contained undetectable levels of phosphorylated  $\beta$ -catenin. Transfection with  $\beta$ -catenin cDNA or inhibition of proteasomal degradation resulted in a fast and extensive increase in p $\beta$ -catenin levels. Interestingly, according to its electrophoretic mobility, the phosphorylated protein is largely non-polyubiquitinated, suggesting that either phosphorylation by GSK-3 $\beta$  is considerably more efficient than the subsequent step in  $\beta$ -catenin turnover, namely ubiquitination, or that excess  $\beta$ -catenin specifically attenuates the ubiquitination process.

As pointed out above, among the cells tested for p $\beta$ -catenin, the colon cancer SW480 cell line was exceptional as it accumulated p $\beta$ -catenin. This result shed new light on the differential regulation by APC of the phosphorylation of  $\beta$ -catenin and its consequent degradation or dephosphorylation. It is well established that the tumor suppressor protein APC and the associated protein axin/conductin provide the scaffold for the phosphorylation of  $\beta$ -catenin (Polakis, 2000) and that  $\beta$ -TrCP is present in a complex with axin and APC (Kitagawa et al., 1999). It is not clear, however, whether APC is playing a role in the actual ubiquitination and degradation of  $\beta$ -catenin. Mutations in APC, which occur in over 80% of human colon cancers, are believed to lead to the accumulation of  $\beta$ -catenin and presumably to the activation of specific genes that confer malignant properties on these cells, for example, Myc (He et al., 1998) and cyclin D1 (Shtutman et al., 1999; Tetsu and McCormick, 1999). It was previously demonstrated that deletion mutants of APC lacking amino acids 1941 to 2644 could not inhibit  $\beta$ -catenin-mediated transactivation in SW480 cells (Morin et al., 1997), suggesting that this domain of APC is necessary for  $\beta$ -catenin downregulation (Morin et al., 1997).

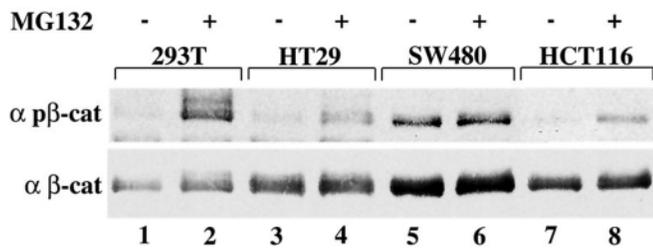


**Fig. 7.** p $\beta$ -catenin is transiently associated with nascent adherens junctions. (a) MDCK cells were transfected with HA- $\beta$ -catenin and after 48 hours fixed and stained as described in the Materials and Methods. Ratio imaging analysis was carried out at six optical sections (0.6  $\mu$ m apart) and shows exclusive localization of p $\beta$ -catenin (red) in nuclear speckles and not in adherens junctions. (b) BCAP cells were plated on coverslips for the specified times (A-D) or treated with 4 mM EGTA for 1 hour (E) or washed and allowed to recover for 3 hours in the presence of fresh medium containing Ca<sup>2+</sup> (F). The cells were fixed, stained and analyzed by ratio imaging analysis. The colors represent the ratio between p $\beta$ -catenin (red) and either HA  $\beta$ -catenin (blue, in the transfected MDCK cells) or total endogenous  $\beta$ -catenin (blue, in the BCAP cells). The insert in A shows the specific inhibition of the p $\beta$ -catenin staining in the presence of the double phosphorylated peptide (pep). Bar, 10  $\mu$ m.

The results presented here indicate that phosphorylation and ubiquitination of  $\beta$ -catenin may be differentially regulated by distinct regions of the APC molecule. Thus, truncation of the APC molecule at position 1338 (like in SW480 cells) can support some phosphorylation, but such APC lacks the region that is necessary for promoting the degradation of  $\beta$ -catenin. Alternatively, these cells may be deficient in  $\beta$ -catenin dephosphorylation, resulting in the accumulation of p $\beta$ -catenin. APC truncated at a more C-terminal position (1555 as found in HT29 cells) can support some phosphorylation, although at a lower level, compared with wild-type APC in 293T cells, but the subsequent ubiquitination and degradation of the molecule are efficient, since the phosphorylated

molecule is detected in these cells only after blocking proteasomal degradation. It thus appears that the region between amino acids 1338 and 1555 of the APC containing the second 20 amino acid repeat region is particularly important for the turnover of p $\beta$ -catenin by either promoting degradation or affecting its dephosphorylation.

A related intriguing observation, highlighted by this study, is the capacity of p $\beta$ -catenin to undergo rapid dephosphorylation and thus avoid proteasomal degradation. According to the common view,  $\beta$ -catenin phosphorylation is largely a unidirectional process, leading to the ubiquitination and degradation of the protein. To test whether  $\beta$ -catenin phosphorylation is a reversible process, we blocked the



**Fig. 8.** p $\beta$ -catenin expression in colon cancer cell lines. HT29, SW480 and HCT116 colon carcinoma cells and 293T cells were either treated (+) with MG132 for 4 hours or left untreated (-). Cell extracts were analyzed by western blotting using both the  $\alpha$ p $\beta$ -catenin antibody and a general anti- $\beta$ -catenin antibody.

phosphorylation of  $\beta$ -catenin by LiCl and its proteasomal degradation by MG132, and monitored the changes in p $\beta$ -catenin levels with the  $\alpha$ p $\beta$ -catenin antibody. Such treatment resulted in a rapid decline in p $\beta$ -catenin levels ( $t_{1/2}$ =10 minutes), suggesting that the molecules can undergo rapid dephosphorylation. The rate of  $\beta$ -catenin dephosphorylation was comparable to that of its phosphorylation, which was an approximately seven-fold decrease or increase in phosphorylated  $\beta$ -catenin after 1 hour of treatment. The regulation of  $\beta$ -catenin levels was commonly attributed to the activation of Wnt signaling, which includes the inhibition of GSK-3 $\beta$  activity that leads to  $\beta$ -catenin accumulation (Zhurinsky et al., 2000b). The results described in this study suggest that dephosphorylation of  $\beta$ -catenin might be an alternative pathway that can protect  $\beta$ -catenin and induce its accumulation, a process equivalent to the activation of Wnt signaling. Whether such process indeed takes place, and what might be the exact activation mechanism, remains to be determined. Effects of dephosphorylation by protein phosphatase 2A (PP2A) in the degradation complex of APC-Axin-GSK3 $\beta$ - $\beta$ -catenin were previously suggested. PP2A was found to dephosphorylate axin following Wnt signaling, thereby releasing  $\beta$ -catenin from the degradation complex (Willert et al., 1999). On the other hand, it has been shown that PP2A can promote the degradation of  $\beta$ -catenin and inhibit Wnt signaling (Li et al., 2001; Seeling et al., 1999; Yamamoto et al., 2001). A dephosphorylation activity, directed to the  $\beta$ -catenin molecule itself, has not yet been described.

Examination of the effect of  $\beta$ -catenin phosphorylation on the interaction with its different molecular partners revealed some intriguing features. As is evident from co-immunoprecipitation experiments, phosphorylated  $\beta$ -catenin binds to LEF1, yet it apparently fails to efficiently form a ternary complex with DNA and is thus probably transcriptionally inactive. The basis for these properties of p $\beta$ -catenin is not clear, but it appears likely that phosphorylation may affect its transactivation capacity. This notion is supported by the observation that mutations in the putative GSK-3 $\beta$  phosphorylation site of  $\beta$ -catenin increase its signaling activity when compared with equal amounts of wild-type  $\beta$ -catenin (Guger and Gumbiner, 2000). In addition a recent finding demonstrated that phosphorylated  $\beta$ -catenin, which accumulates after blocking proteasomal degradation, is unable to activate a synthetic LEF/TCF reporter (TOPFLASH), whereas non-phosphorylated  $\beta$ -catenin accumulating after Wnt or LiCl treatments is transcriptionally active (Staal et al.,

2001). This may suggest that phosphorylation of the N-terminal serines of  $\beta$ -catenin have a regulatory role in  $\beta$ -catenin signaling in addition to its effect on protein stability.

Another intriguing feature of p $\beta$ -catenin is its conspicuous absence from mature cell-cell adherens junctions; yet it has a transient association with newly formed adhesions. The latter observation suggests that p $\beta$ -catenin has the intrinsic capacity to interact with the various junctional partners (e.g. cadherin and/or  $\alpha$ -catenin), yet these interactions can take place only during early stages of junction formation (first several hours after cell plating, or EGTA treatment), when presumably new cytoplasmic  $\beta$ -catenin molecules are recruited to the membrane, but not at later stages, when the turnover of the recruited  $\beta$ -catenin molecules is rather limited. It is not surprising that GSK-3 $\beta$ -mediated phosphorylation does not occur in adherens junctions since  $\beta$ -catenin interacts with junctional cadherin via its arm repeats that are also involved in its association with APC that is essential for  $\beta$ -catenin phosphorylation (Hulsken et al., 1994). The subcellular distribution of the  $\beta$ -catenin destruction complex, including APC, axin, GSK-3 $\beta$  and dishevelled in polarized epithelial cells, is mostly cytoplasmic (Reinacher-Schick and Gumbiner, 2001). In addition, an association of PP2A with the cadherin- $\beta$ -catenin complex was reported (Gotz et al., 2000), suggesting that even when p $\beta$ -catenin forms a complex with cadherin it may be prone to dephosphorylation activity. The putative role and significance of this association of p $\beta$ -catenin with newly formed junctions is still to be determined. The transient localization of  $\beta$ -catenin in junctions or its accumulation in the nuclei may reflect a compartmentalized shelter from degradation, whose physiological significance is also unclear at present.

Taken together the observations of this study suggest that p $\beta$ -catenin is a short lived, transcriptionally inactive intermediate, with a half life determined by the fine-tuned balance between phosphorylation, dephosphorylation, ubiquitination, proteasomal degradation and subcellular localization.

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