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23. We fixed and permeabilized 293T cells in methanol at −20°C for 4 min. Primary antibodies were diluted 1:200 in 10 mM tris-buffered saline containing 1% bovine serum albumin, except for MC5-Cy3, which was used at 1:50 dilution. The secondary conjugates used were Cy2-conjugated donkey anti-mouse IgG (1:200) and Cy3-conjugated donkey anti-rabbit IgG (1:400) (Jackson ImmunoResearch Laboratories, West Grove, PA). Confocal images were acquired on a confocal laser scanning microscope (model LSM410, Carl Zeiss Inc.) equipped with a ×63/1.4Plan-APOCHROMAT oil immersion objective. Each image represents a 2D projection of sections in the Z series, taken across the depth of the cell at 0.5-μm intervals.
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9 October 1998; accepted 23 February 1999

Regulation of β-Catenin Signaling by the B56 Subunit of Protein Phosphatase 2A

Joni M. Seeling,[1] Jeffrey R. Miller,[3] Rosario Gil,[1]* Randall T. Moon,[3] Ray White,[1] David M. Virshup[1,2]†

Dysregulation of Wnt–β-catenin signaling disrupts axis formation in vertebrate embryos and underlies multiple human malignancies. The adenomatous polyposis coli (APC) protein, axin, and glycogen synthase kinase 3β form a Wnt-regulated signaling complex that mediates the phosphorylation-dependent degradation of β-catenin. A protein phosphatase 2A (PP2A) regulatory subunit, B56, interacted with APC in the yeast two-hybrid system. Expression of B56 reduced the abundance of β-catenin and inhibited transcription of β-catenin target genes in mammalian cells and *Xenopus* embryo explants. The B56-dependent decrease in β-catenin was blocked by oncogenic mutations in β-catenin or APC, and by proteasome inhibitors. B56 may direct PP2A to dephosphorylate specific components of the APC-dependent signaling complex and thereby inhibit Wnt signaling.

In vertebrate cells, Wnt, a secreted glycoprotein, regulates growth and development in part by controlling the activity of a heterodimeric transcription factor containing β-catenin and a member of the Lef-Tcf family of high mobility group transcription factors. β-Catenin binds an APC-axin complex, where it is phosphorylated by glycogen synthase kinase 3β (GSK3β) on NH_2-terminal residues. This phosphorylation event results in the ubiquitin-mediated proteasomal degradation of β-catenin. Wnt signaling leads to the inactivation of GSK3β, which results in an accumulation of β-catenin that then binds to Lef-Tcf and activates transcription of target genes (*1*). Mutations in the APC gene that produce a truncated polypeptide unable to promote the degradation of β-catenin are found in 85% of sporadic colon cancers. Point mutations in β-catenin that alter putative GSK3β phosphorylation sites have been reported in colon, pancreatic, hepatic, and skin cancers (*2*).

[1]Department of Oncological Sciences, Huntsman Cancer Institute, and [2]Department of Pediatrics, University of Utah, Salt Lake City, UT 84132, USA. [3]Howard Hughes Medical Institute, University of Washington, Seattle, WA 98195, USA.

*Present address: Departamento de Bioquimica y Biologia Molecular, Universitat de Valencia, Apartado de Correos 73, 46100 Burjassot (Valencia), Spain.
†To whom correspondence should be addressed. E-mail: david.virshup@hci.utah.edu

The APC protein contains a number of predicted protein-protein interaction domains, suggesting that it acts as a scaffold for the assembly of signaling molecules including β-catenin, GSK3β, and axin. Domains in the central third of APC are required for the binding of these molecules. Armadillo and heptad repeat motifs, which are thought to mediate protein-protein interactions, are present in the NH_2-terminal third of the protein. To identify additional APC-interacting proteins that may regulate Wnt signaling, we performed a two-hybrid screen with the NH_2-terminal third of APC. In a screen of a human B cell cDNA library, clones encoding the B56α and B56δ isoforms of the B56 family of PP2A regulatory subunits were isolated (*3*).

PP2A is an intracellular serine-threonine protein phosphatase. It is a heterotrimeric protein containing conserved catalytic (C) and structural (A) subunits, and a variable regulatory (B) subunit. Three unrelated families of PP2A B subunits have been identified to date, denoted B, PR72, and B56 (B′). These B subunits regulate the subcellular localization and substrate specificity of PP2A, and distinct PP2A heterotrimers can dephosphorylate different sites on the same substrate (*4*). The B56 family of PP2A regulatory subunits includes five widely expressed paralogous genes (α, β, δ, ε, and γ) (*5*, *6*). To confirm the interaction of APC and B56, we tested plasmids encoding a number of PP2A subunits for interaction with APC in a directed two-hybrid assay (*7*). APC interacted with all assayed members of the B56 family, whereas there was no detectable interaction with Bα (a member of the B family), PR72, A, or C subunits (*8*).

One essential function of the Wnt pathway signaling complex is to regulate the abundance of β-catenin. Multiple phosphorylation events occur in this complex, including the phosphorylation of APC, GSK3β, β-catenin, and axin (*9*). The possible interaction of a PP2A regulatory subunit with this complex indicates that phosphatase activity may regulate β-catenin signaling. To test this, we assessed the effect of okadaic acid, a cell-permeable serine-threonine phosphatase inhibitor, on β-catenin abundance. Treatment of HEK 293 cells with okadaic acid caused an increase in the abundance of β-catenin (Fig. 1A). This suggests that if a PP2A heterotrimer participates in the Wnt–β-catenin pathway, it is likely to be inhibitory to the signaling process. We therefore determined whether increased expression of B56 had any effect on the amounts of β-catenin in 293 cells. Expression of B56α decreased the amount of β-catenin (Fig. 1B). Expression of other members of the B56 family also reduced the abundance of β-catenin (Fig. 1C). The down-regulation of β-catenin was specific to the B56 family, however, because transfection of empty vector, or expression of Bα, had no effect on the amount of β-catenin.

The degradation of phosphorylated β-catenin can be blocked by proteasome inhibitors (*10*). We therefore transfected 293 cells with vectors encoding hemagglutinin (HA)-tagged B56α and either Myc-tagged β-catenin (Fig. 2A) or untagged β-catenin (Fig. 2B) and then treated the transfected cells with the proteasome inhibitors MG-132 (Fig. 2A) or *N*-acetyl-Leu-Leu-norleucinal (ALLN) (Fig. 2B). In both instances, the B56-induced decrease in β-catenin abundance was blocked by the inclusion of the proteasome inhibitor. The peptide aldehyde calpain inhibitor *N*-acetyl-Leu-Leu-methional (ALLM) had no effect on the B56-induced degradation of β-catenin (*8*).

Deletion of the first 90 amino acids of β-catenin (Δ90β-catenin) that encompass the putative GSK3β phosphorylation sites produces a stable protein that accumulates in the

cell (*11*). To determine if B56 expression regulated the abundance of β-catenin through a mechanism that required this domain, we transfected plasmids expressing HA-tagged B56α into 293 cells with plasmids expressing either full-length or Δ90β-catenin. Although expression of B56α led to a decrease in the amount of full-length β-catenin (Fig. 2B), it had no effect on the amount of Δ90β-catenin. The results with Δ90β-catenin and with proteasome inhibitors, taken together, indicate that expression of B56 increases the phosphorylation-induced proteasomal degradation of β-catenin.

The B56-containing PP2A heterotrimer could function by dephosphorylating a component of the Wnt signaling complex. GSK3β is one potential target of the phosphatase, as PP2A has previously been shown to dephosphorylate and hence activate this kinase in vitro (*12*). Increased GSK3β activity in 293 cells leads to decreased β-catenin accumulation (Fig. 2C). Coexpression of B56 and GSK3β results in the virtual absence of β-catenin, consistent with a model in which PP2A dephosphorylates and activates GSK3β in the APC-axin complex.

Many colon cancer cell lines produce a mutant, truncated APC protein that does not promote the degradation of β-catenin. If B56 regulates the abundance of β-catenin through an interaction with an APC-dependent signaling complex, expression of B56 should not decrease amounts of β-catenin in cell lines expressing only truncated APC. We therefore transfected HCA7 and SW480 colon cancer cell lines (which express wild-type and truncated APC, respectively) and 293 cells (which express wild-type APC) with vectors encoding Myc-tagged β-catenin and HA-tagged B56α (or empty vector). Expression of B56α led to a decrease in the amount of β-catenin in 293 and HCA7 cell lines, but not in the SW480 cell line that lacks full-length APC (Fig. 3). These results suggest that the effect of B56 on β-catenin stability requires intact APC protein.

Although β-catenin has several functions

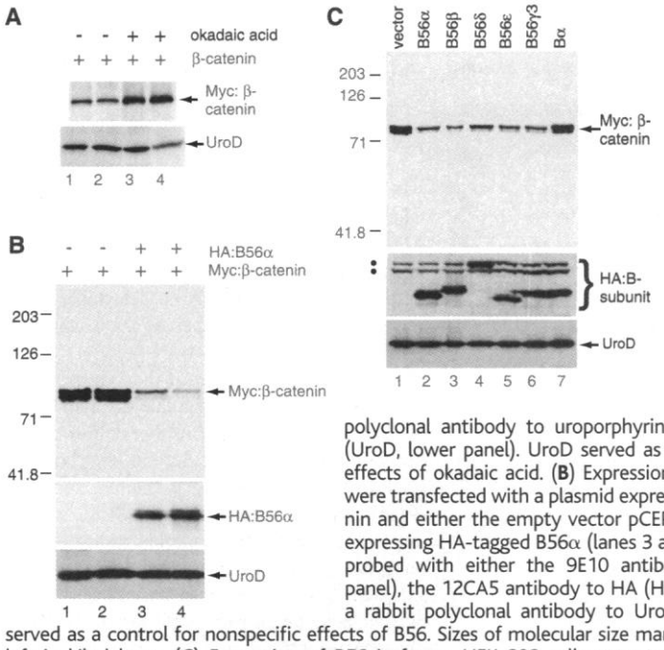

Fig. 1. Effects of PP2A activity on the abundance of β-catenin. (**A**) Treatment with okadaic acid. HEK 293 cells were transfected as described (*18, 19*). Cell cultures were treated with 20 nM okadaic acid (lanes 3 and 4) or vehicle (lanes 1 and 2) for 20 hours. Protein was prepared as described (*20, 21*). Blots were probed with either the 9E10 antibody to Myc (Myc:β-catenin, upper panel) or a rabbit polyclonal antibody to uroporphyrinogen decarboxylase (*22*) (UroD, lower panel). UroD served as a control for nonspecific effects of okadaic acid. (**B**) Expression of B56α. HEK 293 cells were transfected with a plasmid expressing Myc-tagged β-catenin and either the empty vector pCEP (lanes 1 and 2) or pCEP expressing HA-tagged B56α (lanes 3 and 4). Immunoblots were probed with either the 9E10 antibody (Myc:β-catenin, top panel), the 12CA5 antibody to HA (HA:B56α, middle panel), or a rabbit polyclonal antibody to UroD (bottom panel). UroD served as a control for nonspecific effects of B56. Sizes of molecular size markers are indicated on the left in kilodaltons. (**C**) Expression of B56 isoforms. HEK 293 cells were transfected with a plasmid expressing Myc-tagged β-catenin and either pCEP (lane 1), or pCEP expressing HA-tagged B56α (lane 2), B56β (lane 3), B56δ (lane 4), B56ε (lane 5), B56γ3 (lane 6), or Bα (lane 7). Immunoblots were probed with either the 9E10 antibody (Myc:β-catenin, top panel), the 12CA5 antibody (HA:B-subunit, middle panel), or a rabbit polyclonal antibody to UroD (UroD, bottom panel). Two nonspecific bands on the HA immunoblot, the upper one of which comigrates with HA:B56δ (lane 4), are marked with closed circles.

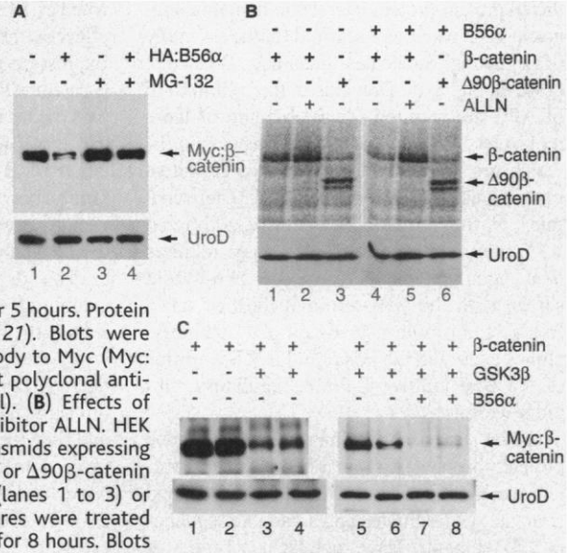

Fig. 2. Inhibition of proteasomal degradation of β-catenin abrogates, whereas the addition of GSK3β potentiates, B56-induced reduction in the abundance of β-catenin. (**A**) Effects of proteasome inhibitor MG-132. HEK 293 cells were transfected with plasmids expressing Myc:β-catenin (all lanes) and either pCEP (lanes 1 and 3) or HA:B56α (lanes 2 and 4). Cell cultures were treated with 20 μM MG-132 (lanes 3 and 4) or vehicle (lanes 1 and 2) for 5 hours. Protein was prepared as described (*20, 21*). Blots were probed with either the 9E10 antibody to Myc (Myc: β-catenin, upper panel), or a rabbit polyclonal antibody to UroD (UroD, lower panel). (**B**) Effects of Δ90β-catenin and proteasome inhibitor ALLN. HEK 293 cells were transfected with plasmids expressing β-catenin (*23*) (lanes 1, 2, 4, and 5) or Δ90β-catenin (lanes 3 and 6) and either pCEP (lanes 1 to 3) or HA:B56α (lanes 4 to 6). Cell cultures were treated with 25 μM ALLN (lanes 2 and 5) for 8 hours. Blots were probed with either antibody to β-catenin and visualized with ^{125}I-labeled antibody to mouse immunoglobulin using a Phosphorimager (Molecular Dynamics) (upper panel) or a rabbit polyclonal antibody to UroD and visualized with ECL (lower panel). Transfection efficiency in these experiments was ~75%, and the full-length β-catenin seen in lanes 1, 2, 4, and 5 reflects that from transfected and nontransfected cells. The β-catenin seen in (A) is Myc-tagged and represents the β-catenin present solely in transfected cells. Therefore, the magnitude of the change in amount of β-catenin caused by B56α appears smaller in (B) than in (A). (**C**) Effects of GSK3β overexpression. HEK 293 cells were transfected as described (*18, 24*) with plasmids expressing GSK3β (lanes 3 to 8) and B56 (lanes 7 and 8). Protein was prepared as described (*20, 21*). Blots were probed with either the 9E10 antibody (Myc:β-catenin, upper panel) or a rabbit polyclonal antibody to UroD (UroD, lower panel).

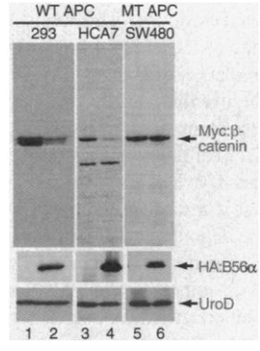

Fig. 3. Absence of B56-mediated decrease in the abundance of β-catenin in APC mutant cells. HEK 293, HCA7, and SW480 cell lines were transfected (*18*) with a plasmid expressing Myc-tagged β-catenin and either pCEP (lanes 1, 3, and 5) or pCEP containing B56α (lanes 2, 4, and 6). Blots were probed with either the 9E10 antibody to Myc (Myc:β-catenin, top panel), the 12CA5 antibody to HA (HA:B56α, middle panel), or a rabbit polyclonal antibody to UroD (UroD, bottom panel). WT, wild-type; MT, mutant.

in the cell, its ability to activate transcription is critical to its role in growth and development. To determine whether the observed reduction in the abundance of β-catenin led to a decrease in its transcriptional activity, we measured β-catenin–dependent transcription from a Lef-1:luciferase reporter in the absence and presence of overexpressed B56α. Expression of B56α reduced luciferase activity by about 50% (Fig. 4A). Expression of Bα had no effect (*8*). Expression of B56α in cell lines with mutant β-catenin or mutant APC resulted in no change in luciferase activity (HCT116 and SW480, respectively, Fig. 4A). Thus, overexpression of B56α causes decreased transcription through β-catenin–dependent promoters. Additionally, the finding that β-catenin and APC mutations block the effects of B56 expression suggests that B56 acts upstream of β-catenin and APC.

Transcriptional targets of β-catenin include genes that specify the dorsal-ventral axis in *Xenopus laevis*. To determine whether B56 can regulate transcription of β-catenin target genes in a heterologous system, we examined the effect of B56α expression on the induction of the siamois and Xnr-3 genes, two β-catenin target genes, in *Xenopus* animal cap explants from RNA-injected embryos. Expression of Xwnt-8 RNA in explants increased expression of siamois and Xnr-3 (Fig. 4B), whereas a green fluorescent protein (GFP) control RNA and B56α alone had no effect. Expression of B56α with Xwnt-8 decreased the response of explants to Xwnt-8. Thus, B56 may function downstream of Wnt in the signaling pathway to β-catenin.

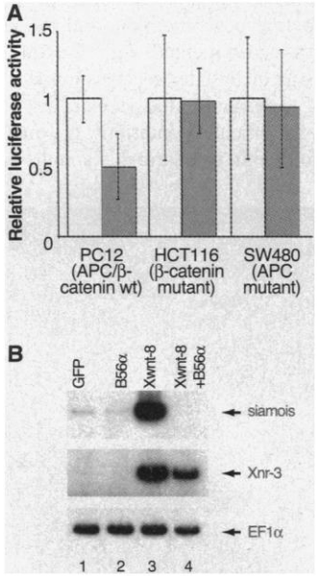

Fig. 4. Effects of B56 on Wnt signaling activity. (**A**) Reduced Lef-1 reporter activity after expression of B56α in cell lines with intact Wnt signaling. PC12, HCT116, and SW480 cell lines were transfected (*18*) with a Lef-1:luciferase reporter (H4WTtk100) and either pCEP (open bars), or pCEP expressing HA:B56α (closed bars) (*25*). The data are represented as the average ± SD from three (HCT116) or four (PC12 and SW480) independent experiments. (**B**) Effects of B56α on the signaling activity of Xwnt-8 in *Xenopus* animal cap explants. Animal poles of two-cell stage embryos were injected with RNA encoding the indicated protein [GFP (lane 1), B56α (lane 2), Xwnt-8 (lane 3), or Xwnt-8 + B56α (lane 4)]. Animal caps were cut at stage 8-9. RNA preparation and reverse transcriptase–polymerase chain reaction were then carried out to determine the expression levels of the transcripts encoding siamois, Xnr-3, and EF1α as described (*26*). EF1α served as a control for the reverse transcription reaction and gel loading. Dose of RNAs injected: GFP, 500 pg; B56α, 500 pg; and Xwnt-8, 125 pg.

Our study suggests that PP2A heterotrimers containing the B56 regulatory subunit function in the Wnt signaling complex to down-regulate β-catenin, perhaps through an interaction of B56 and the NH_2-terminus of APC. The PP2A C subunit has recently been reported to interact with axin, strengthening the conclusion that PP2A is a regulator of Wnt signaling (*13*). Oncogenic mutations in the Wnt pathway, including increased Wnt expression, loss of APC-dependent signaling complexes, and mutation of phosphorylation sites in β-catenin, can lead to increases in β-catenin signaling. Loss of PP2A function may provide an additional route to activation of Wnt signaling and oncogenesis. Consistent with this, mutations in the gene encoding the β isoform of the PP2A A subunit have been identified in colon and lung cancers (*14*). Inhibition of PP2A activity can contribute both to oncogenesis and aberrant development. For example, okadaic acid is a tumor promoter in mice, DNA tumor virus small t antigens inhibit PP2A activity and lead to cellular transformation, the SET and Hox11 protein inhibitors of PP2A are overexpressed in acute leukemias, and B56 is essential for embryonic development in *Caenorhabditis elegans* (*15*, *16*). PP2A appears to counteract multiple growth-promoting signal transduction pathways. The role of the PP2A B56 regulatory subunit may be to direct dephosphorylation, and hence regulate the activity, of specific components of the Wnt signaling complex.

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18. Transfections were done with Lipofectamine (PC12 and HEK 293), Lipofectamine Plus [SW480 (in assessing β-catenin abundance)], Lipofectin [SW480 (in reporter assays) and HCT116], or Lipofectin Plus (HCA7) lipid reagents (Gibco-BRL).
19. Cells were transfected with plasmids expressing Myc:β-catenin, Myc:axin, and Flag:GSK3β.
20. Cells were harvested 48 hours after transfection, homogenized in CAT buffer [50 mM tris-HCl (pH 7.4), 0.5 mM NaF, 0.1 mM sodium vanadate, 2× Complete protease inhibitors (Boehringer)], and centrifuged at 2000*g*. Proteins in the supernatant were quantitated by the Bradford assay. Proteins were separated on a 7.5 % polyacrylamide gel and transferred to Immobilon P (Millipore). Immunoblots were visualized with horseradish peroxidase–conjugated antibody to mouse immunoglobulin G and immunoglobulin M (Pierce) and enhanced chemiluminescence (ECL, Amersham), unless stated otherwise.
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23. β-Catenin and Δ90β-catenin were subcloned from pRETRO vectors into pEF as Bam HI cassettes.
24. Cells were transfected with plasmids expressing Myc:β-catenin, Myc:axin, and XWnt-8.
25. As a control, a mutant Lef-1:luciferase reporter (H4Mtk100) was substituted for the Lef-1 reporter. All transfections included a cytomegalovirus:β-galactosidase construct to control for transfection efficiency. Lef-1–dependent activity was defined as the ratio of activity from the wild-type Lef-1:luciferase reporter plasmid divided by luciferase activity from the mutant Lef-1 reporter. This value was normalized to Lef-1 reporter activity in the absence of B56 expression. Luciferase activity was determined with luciferase reagent (Promega) and β-galactosidase was determined with GALACTON reagent (Tropix).
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27. We thank S. Elledge, M. Waterman, P. Polakis, D. Pallas, J. Goris, B. Hemmings, J. Phillips, N. Matsunami, and A. Thorburn for reagents and members of the Virshup laboratory. Supported by the Huntsman Cancer Institute, Cancer Training grant T32CA09602 to J.M.S., and R01 CA71074 to D.M.V. Oligonucleotide synthesis was supported by Cancer Center grant 3P30CA42014. J.R.M. is an Associate and R.T.M. an Investigator of the Howard Hughes Medical Institute, which supported their contributions.

19 October 1998; accepted 10 February 1999