Protein Phosphatase 2Cα Dephosphorylates Axin and Activates LEF-1-dependent Transcription*

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The Dishevelled (Dvl) gene family encodes cytoplasmic proteins that are necessary for Wnt signal transduction. Utilizing the yeast two-hybrid system, we identified protein phosphatase 2Cα (PP2C) as a Dvl-PDZ domain-interacting protein. PP2C exists in a complex with Dvl, β-catenin, and Axin, a negative regulator of Wnt signaling. In a Wnt-responsive LEF-1 reporter gene assay, expression of PP2C activates transcription and also elicits a synergistic response with β-catenin and Wnt-1. In addition, PP2C expression relieves Axin-mediated repression of LEF-1-dependent transcription. PP2C utilizes Axin as a substrate both in vitro and in vivo and decreases its half-life. These results indicate that PP2C is a positive regulator of Wnt signal transduction and mediates its effects through the dephosphorylation of Axin.

Wnt genes are differentially regulated during development and encode secreted glycoproteins that are involved in cell signaling, cell fate determination, and oncogenesis (1, 2). The current model of Wnt signal transduction proposes that in the absence of a Wnt signal, β-catenin binds an APC1-Axin complex, where it is phosphorylated by GSK-3β and subsequently targeted for ubiquitin-mediated proteasomal degradation. When Wnt signaling is activated via ligand binding to the frizzled encoded receptors, the activity of GSK-3β is inhibited, and cytosolic β-catenin accumulates and interacts with TCF/LEF-1 transcription factors, leading to transcription to the nucleus and transcriptional modulation of target genes (3). Axin, the product of the mouse fused locus, has been identified as a negative regulator of the Wnt signaling pathway and is closely related to Axil/conductin protein (4–7). Several studies demonstrated that Axin and Axil/conductin simultaneously bind to GSK-3β and β-catenin (7–9). Furthermore, the regulators of the G protein signaling (RGS) domain of Axin directly interacts with APC, with expression of Axin resulting in the rapid turnover of β-catenin (6, 10, 11).

The dishevelled gene family (dsh/Dvl) encodes cytoplasmic proteins that are essential for transmission of the wg/Wnt signal. Although no biochemical function has been identified, all dishevelled proteins contain three highly conserved domains: an amino-terminal DIX domain, a central PDZ domain, and a carboxyl-terminal DEP domain (12–14). The DIX and PDZ domains have been shown to be essential for wg/Wnt signaling, whereas the DEP domain is required for planar cell polarity signaling in Drosophila and activation of the Jun-NH2-terminal kinase pathway in fly and mammalian systems (15–19). The DIX and PDZ domains have been shown to be involved in Dvl/Axin interaction (20–22).

EXPERIMENTAL PROCEDURES

Yeast Two-hybrid System—The two-hybrid screen and X-gal colony filter assays were performed as described (23). The hybrid bait was constructed by fusing the region encoding the Dvl3 PDZ domain (amino acids 186–391) to the LexA DNA-binding domain in pEG202 (provided by R. Brent, Harvard Medical School, Boston, MA). A human fetal brain cDNA library expressed from pJG4–5 (provided by D. Krainc, Harvard Medical School) was screened, and interactions were detected in the yeast strain EGY48 (matα ura3 trp1 his3 3LexAop-Leu2) based on galactose-dependent growth on SD/His/−/−Trp medium containing 2 mM IPTG. The expression plasmid was kindly provided by R. Costantini (Columbia University, New York) and A. McMahon (Harvard Medical School, respectively). The LEF-1 and LEF/luciferase expression plasmids were generously provided by R. Grosschedel (University of California, San Francisco). The tau expression plasmid was kindly provided by M. Monette (University of Maryland, Baltimore).

Transfections in COS-1 cells were performed using SuperFect transfection reagent (Qiagen). COS-1 cells (6 × 105) were incubated for 3 h with SuperFect reagent-DNA complexes, with the optimal ratio determined to be 30 μl of SuperFect reagent/5 μg of DNA. The total amount of DNA for each transfection was kept constant by the addition of the empty expression vector pCS2+. Cells were harvested 24 h after transfection.

Immunoprecipitations—Transfected COS-1 cells were resuspended in 500 μl of RIPA buffer (50 mM Tris, pH 7.5, 150 mM NaCl, 0.5% deoxycholate, 1% Nonidet P-40, 1 mM NaF, 1 mM Na3VO4, 1 mM sodium orthovanadate) containing the protease inhibitor mixture Complete (Roche Molecular Biochemicals) and incubated for 20 min on ice. Cell debris was removed by centrifugation, and lysates were precleared with protein G-Sepharose (Amersham Pharmacia Biotech). Cell lysates (150 μg) were immunoprecipitated with antibodies to PP2Cα (Upstate Biotechnology) using standard procedures. Immunoprecipitates were washed five times in RIPA buffer containing 0.05% SDS and analyzed by SDS-PAGE.

Antibodies and Western Blot Analysis—Mouse monoclonal antibodies were generated against a glutathione S-transferase (GST) fusion protein of PDE5A3 (Upstate Biotechnology) using standard procedures. Immunoprecipitates were washed five times in RIPA buffer containing 0.05% SDS and analyzed by SDS-PAGE.

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tein containing the 100 carboxyl-terminal amino acids of Dvl3 using the ClonCell™-HY hybridoma cloning kit (Stem Cell Technologies, Inc.). Hybridoma supernatants were initially screened by enzyme-linked immunosorbent assay against the fusion protein, with positive clones undergoing a second screen against GST to eliminate those recognizing the GST portion of the fusion protein. The supernatants from positive clones were subsequently screened against GST and the fusion protein by Western blot analysis. Hybridoma clone 3-4D3 displayed the highest level of reactivity and was specific for Dvl3.

Transiently transfected COS-1 cells were resuspended in 3× Læmmlí sample buffer, boiled for 5 min, and analyzed by SDS-PAGE. Immunoblot analysis was performed with anti-Dvl3, anti-β-catenin (Transduction Labs), anti-PP2C (Upstate Biotechnology), anti-tau C-17 (Santa Cruz Biotechnology), or anti-Myc-horseradish peroxidase (Invitrogen) antibodies using standard procedures. Blots were visualized with the ECL Plus detection system (Amersham Pharmacia Biotech).

**Reporter Gene Assay**—Cells were transfected with 2.0 or 1.5 µg of each expression plasmid, 1.0 µg of luciferase reporter plasmid, 0.5 µg of LEF-1 expression plasmid, and 0.5 µg of PCMβ-β-galactosidase expression vector as an internal control. Luciferase and β-galactosidase activity were measured 24 h after transfection using reagents from Promega according to the manufacturer’s instructions. A total of 20 µg of protein was used for luciferase determination, except for samples from β-catenin transfected cells, in which case 0.5 µg was used. The relative luciferase activities presented were normalized by dividing the luciferase activity by the β-galactosidase activity.

**In Vitro Phosphatase Reaction**—COS-1 cells (6 × 10⁵) transfected with the Myc-Axin expression construct were lysed in RIPA buffer containing 0.1% SDS, and cell lysates (150 µg) were immunoprecipitated with 1.2 µg of anti-Myc antibody A-14 (Santa Cruz Biotechnology). Immune complexes were recovered with protein G-Sepharose™ and washed four times with RIPA buffer containing no phosphatase inhibitors and twice in phosphate reaction buffer (50 mM Tris, pH 7.5, 0.1 mM EDTA, pH 8.0, 0.1% 2-mercaptoethanol) in the presence or absence of 60 mM magnesium acetate.

Immunoprecipitates were resuspended in 20 µl of phosphate reaction buffer containing 0.64 µg of PP2Cv2 (Upstate Biotechnology) in the presence of 60 mM magnesium acetate, or in the presence of 60 mM magnesium acetate and 2.5 mM okadaic acid, and incubated at 30 °C for 30 min. Reactions were terminated by the addition of 15 µl of 3× Læmmlí sample buffer, boiled for 5 min, and analyzed by SDS-PAGE.

**Pulse-Chase Analysis**—COS-1 cells (2 × 10⁶) were seeded in 35-mm dishes and transfected with 1 µg of Myc-Axin and 4 µg of either pcS2 or pcS2-PP2C using SuperFect transfection reagent (Qiagen). After 24 h, pulse-chase analysis was performed essentially as described (24). Briefly, transfected COS-1 cells were preincubated for 15 min in medium lacking methionine and cysteine prior to labeling. After labeling for 30 min, plates were rinsed twice with phosphate-buffered saline and 0.5 ml of lysis buffer (50 mM Tris (pH 7.5) 150 mM NaCl, 0.5% deoxycholate, 1% Nonidet P-40, 0.1% SDS, 1 mM NaVO₄, 1 mM NaF, 1 mM calycin A, and protease inhibitor mixture Complete™ (Roche Molecular Biochemicals)). Cell lysates were preclarified for 1 h using protein G-Sepharose and incubated overnight with anti-Myc epitope antibody. Immune complexes were recovered with protein G-Sepharose™, washed three times with lysis buffer, suspended in 30 µl of Læmmlí sample buffer and boiled for 3 min. Samples were analyzed by 6% SDS-PAGE followed by autoradiography.

**RESULTS AND DISCUSSION**

To understand the molecular mechanisms of Dvl function, we searched for new interacting proteins with Dvl. A yeast two-hybrid screen of a human fetal brain cDNA library conditionally expressed from the GAL1 promoter identified three positive clones that interacted with the LexA-Dvl2 PDZ domain bait construct. The specificity of these interactions was assessed using the colony color filter assay for β-catenin and recent evidence demonstrating a direct interaction between Dvl1 and Axin (20, 22). Taken together, these results demonstrate that PP2C associates with β-catenin and suggest that these proteins exist in a complex with β-catenin.

**β-Catenin has been shown to interact with LEF-1 DNA binding factors and regulate transcription (10). To ascertain a role for PP2C in Wnt signaling, we analyzed the effect of PP2C on LEF-1-mediated transcription of a luciferase reporter gene. A CMV-driven β-galactosidase construct was utilized as an internal control for normalization of luciferase activities. Whereas expression of PP2C had no effect on β-galactosidase activity**

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*Fig. 1. PP2C interacts with Dvl3, β-catenin, and axin.* A, cell lysates (150 µg of protein) from COS-1 cells co-transfected with plasmids encoding PP2C and Dvl3 (top and second panel) or PP2C and Myc-Axin (third and fourth panel) were immunoprecipitated with (+) or without (−) anti-PP2C antibodies. Immunoprecipitates were resolved by SDS-PAGE and analyzed by Western blotting using anti-Dvl3 (top panel), anti-β-catenin (second panel), anti-Myc epitope (third panel), or anti-PP2C (bottom panel) antibodies. 10 µg of precleared cell lysate obtained from each sample prior to immunoprecipitation was also loaded to confirm the position of Dvl3, β-catenin, and Myc-Axin (Lysate). B, cell lysates from COS-1 cells co-transfected with PP2C and Myc-Axin were immunoprecipitated with anti-Myc epitope (+) or control antibodies (−). The Western blot was probed with anti-PP2C (top panel) and anti-Myc epitope antibodies (middle panel). The bottom panel displays IgG light chain as a control for equal loading.

PP2C, a magnesium-dependent serine/threonine phosphatase (26). All three proteins also interacted in the yeast system with the PDZ domain of Dvl1 (data not shown), consistent with the high level of sequence conservation among the Dvl proteins.

Initial attempts to co-immunoprecipitate FLAG epitope-tagged constructs of CW-1 and β-filamin with Dvl3 were unsuccessful, and we focused therefore on our third positive clone, PP2C. To confirm the interaction between PP2C and Dvl3 in mammalian cells, we co-expressed PP2C and Dvl3 in COS-1 cells by transient transfection and analyzed immunoprecipitates by Western blot. When PP2C and Dvl3 were co-expressed in COS-1 cells, an anti-PP2C polyclonal antibody immunoprecipitated Dvl3 protein (Fig. 1A). This antibody also co-immunoprecipitated endogenous β-catenin as determined by Western blot analysis (Fig. 1B). Additionally, we found that Dvl3 and β-catenin co-immunoprecipitated using antibodies against either β-catenin or Dvl3. Previously, it had been shown that Axin binds β-catenin to regulate its abundance (9). Based on our observations that PP2C associates with β-catenin and recent evidence demonstrating a direct interaction between Dvl1 and Axin (20, 22), we explored the possibility of a physical interaction between PP2C and Axin. When lysates from COS-1 cells transiently transfected with PP2C and Myc epitope-tagged Axin were immunoprecipitated with antibodies to PP2C, Myc-Axin was detected in the immune complexes (Fig. 1A). Likewise, PP2C was brought down with immunoprecipitated Myc-Axin (Fig. 1B). Taken together, these results demonstrate that PP2C associates with both Dvl and Axin and suggest that these proteins exist in a complex with β-catenin.

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*2 D. Song, D. J. Sussman, D. and Seldin, unpublished results.*
PP2C Activates LEF-1-dependent Transcription

Fig. 2. Effects of PP2C and PP2CR/K on LEF-1-mediated transcription. A–D, COS-1 cells were transiently transfected with the LEF-1-luciferase reporter, pCMV β-galactosidase expression vector, and 2.0 (A–C) or 1.5 µg (D) of the indicated plasmids in the absence (stippled bars) or presence (shaded bars) of LEF-1 expression plasmid. RLU, relative luciferase units. A, PP2C stimulates LEF-1-mediated transcription. PP2C and PP2CR/K were shown to have equal expression levels by Western blot analysis of cell lysates (data not shown). B and C, co-expression of PP2C, but not PP2CR/K, enhances activation of the LEF-1 reporter gene by β-catenin (B), Dvl3 (C), and Wnt-1 (C). D, PP2C relieves Axin-mediated repression of LEF-1 reporter activity. Each assay was performed in duplicate, and the data represent the average ± S.D. from two independent experiments.

(data not shown), the expression of PP2C and LEF-1 in COS-1 cells resulted in a marked increase in luciferase activity (Fig. 2A), demonstrating activation of the Wnt pathway. Moreover, co-expression of PP2C with Wnt-1, Dvl3, or β-catenin enhanced LEF-1 reporter activity 1.75-, 1.81-, and 1.70-fold, respectively, when compared with cells expressing Wnt-1, Dvl3, or β-catenin alone (Fig. 2, B and C). In an analysis of five independent experiments, the inclusion of PP2C resulted in a synergistic effect, as the average fold induction over the minus LEF-1 controls by PP2C or Wnt alone was 8.40 and 9.01, respectively, whereas co-expression caused a 25.40-fold induction (data not shown). Only low levels of transcription of the LEF-1 reporter were observed in the absence of co-expressed LEF-1, indicating that PP2C, Wnt-1, Dvl3, and β-catenin mediate their effects on reporter gene transcription through LEF-1. A catalytically deficient PP2C mutant, PP2CR/K, was generated to verify that phosphatase activity is required for activation of the LEF-1 reporter gene. This mutation, in which the conserved Arg<sup>195</sup> is altered to Lys, has been shown to confer reduced phosphatase activity in the Caenorhabditis elegans homolog FEM-2 without affecting the overall structure and stability of the protein (27). PP2CR/K demonstrated some residual activity, as evidenced by transcriptional activation of the LEF-1 reporter gene; however, luciferase activities were approximately 47% lower than those obtained in COS-1 cells expressing wild-type PP2C (Fig. 2A). In addition, co-expression of PP2CR/K with Wnt-1, Dvl3, or β-catenin failed to significantly enhance reporter gene activity. Similar results were obtained with a second mutant, PP2CR/A, in which the conserved Arg<sup>195</sup> is altered to Ala (data not shown).

Axin has been identified as a negative regulator of Wnt signaling (4). To determine whether the interaction of PP2C and Axin affects the ability of Axin to inhibit Wnt signal transduction, the LEF-1 reporter gene assay was performed. As seen in previous studies (8), expression of Axin alone failed to activate LEF-1 reporter gene transcription, and co-expression of Axin with Wnt-1 inhibited the effects of Wnt-1 on LEF-1 reporter activity (Fig. 2D). Co-expression of PP2C with Wnt-1 and Axin, however, restored transcription levels to those obtained with Wnt-1 alone (Fig. 2D), whereas PP2CR/K had no effect (Fig. 2D).

Previous studies in Drosophila and mammalian cell lines have shown that a hyperphosphorylated, membrane-associated form of dsh/Dvl is generated in response to wgo/Wnt signaling (15, 28, 29). A number of signaling molecules are activated when translocated to the plasma membrane, suggesting that the hyperphosphorylated form of dsh/Dvl represents an active form of the protein involved in mediating the wgo/Wnt signal (15). We therefore investigated the possibility that PP2C acts as a negative regulator of the Wnt pathway by dephosphorylating Dvl. To determine whether Dvl3 was a substrate for PP2C, we assessed the phosphorylation status of Dvl3 by examining the effect of PP2C on its electrophoretic mobility. We observed no change in the migration or relative composition of the hyper- or hypophosphorylated forms of transfected Dvl3 in the presence of co-transfected PP2C (data not shown), suggesting that Dvl3 is not the direct target of PP2C.

We next examined the effect of PP2C on the activity of GSK-3β, a serine/threonine kinase that negatively regulates the Wnt pathway through the phosphorylation of β-catenin (30). Our results from the LEF-1 reporter assay indicate that PP2C activates Wnt signal transduction. Based on these observations, we reasoned that PP2C might function to inhibit the kinase activity of GSK-3β, thereby promoting the Wnt signal. We monitored the effect of PP2C on GSK-3β-mediated phosphorylation of tau (31) in COS-1 cells. Western blot analysis of tau-transfected COS-1 cells using a phosphorylation-independent tau antibody revealed that tau migrated as a doublet (Fig. 3A). Consistent with previous observations (32), co-transfection of tau with GSK-3β resulted in the phosphorylation of tau by GSK-3β, reducing the electrophoretic mobility of the tau protein (Fig. 3A). Co-expression of PP2C with tau and GSK-3β did
Axin could serve as a direct substrate for PP2C. Western blot analysis of Axin immunocomplexes treated with PP2C resulted in a shift in the electrophoretic mobility of Axin that was not observed in untreated samples (Fig. 3C). Moreover, the dephosphorylation of Axin by PP2C was dependent on the presence of Mg$^{2+}$ (Fig. 3C), consistent with the known properties of PP2C enzymes (26). Addition of okadaic acid, a potent inhibitor of type 1, 2A, and 2B phosphatases (34), did not prevent the observed dephosphorylation of Axin (Fig. 3C), providing further evidence that PP2C is the enzyme mediating this effect. Taken together, these results suggest that PP2C functions to relieve Axin’s inhibition of Wnt-1 signaling through the dephosphorylation of Axin.

A recent study determined that the phosphorylation of Axin is important for its stability (24). To determine whether PP2C expression has an effect on Axin half-life, we performed pulse-chase experiments on COS-1 cells co-transfected with vectors expressing a Myc-tagged Axin and PP2C. As shown in Fig. 4, the expression of PP2C leads to a significant decrease in half-life of the Myc-tagged Axin.

The reversible phosphorylation of proteins is a fundamental mechanism in signal transduction systems that regulate a variety of cellular events, and the important role of protein phosphatases in this process is well documented (35). We identified PP2C as a new potential component of the Wnt signal transduction pathway and have demonstrated its ability to function as a positive regulator of Wnt signaling possibly through interactions with Dvl and Axin. Axin contains several predicted sites for serine/threonine phosphorylation and is directly phosphorylated by GSK-3β (9). Although the phosphorylation status of Axin does not affect its binding to GSK-3β and β-catenin, Axin is important for its stability (24). To determine whether PP2C expression has an effect on Axin half-life, we performed pulse-chase experiments on COS-1 cells co-transfected with vectors expressing a Myc-tagged Axin and PP2C. As shown in Fig. 4, the expression of PP2C leads to a significant decrease in half-life of the Myc-tagged Axin.
protein (24). In addition, the phosphorylation of APC and β-catenin by GSK-3β has been demonstrated to be critical for the rapid turnover of β-catenin by the ubiquitination- proteosome pathway. Our results demonstrate that PP2C dephosphorylates Axin and propagates the Wnt signal; however, the biochemical mechanisms regulating this process, as well as the precise role of the association between PP2C and Dvl, remains unclear. The activation of Wnt signaling by Dvl and PP2C is interesting in light of the findings that these proteins have opposing effects on the regulation of the Jun-NH₂-terminal kinase pathway (18, 19, 36, 37).

Recently, the catalytic subunit of PP2A was shown to interact with Axin (38), whereas the PP2A B56 regulatory subunit interacts with APC and β-catenin (39). Interestingly, in contrast to PP2C, PP2A was found to be inhibitory to Wnt signaling, as expression of the B56 subunit reduced the abundance of β-catenin and decreased its transcriptional activity (39). This report provides strong evidence for a role for PP2C in the activation of the Wnt signal transduction pathway.

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