Inhibition of the Wnt Signaling Pathway by the PR61 Subunit of Protein Phosphatase 2A*

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Axin, a negative regulator of the Wnt signaling pathway, forms a complex with glycogen synthase kinase-3β (GSK-3β), β-catenin, adenosomatous polyposis coli (APC) gene product, and Dvl, and it regulates GSK-3β-dependent phosphorylation in the complex and the stability of β-catenin. Using yeast two-hybrid screening, we found that regulatory subunits of protein phosphatase 2A, PR61β and γ, interact with Axin. PR61β or γ formed a complex with Axin in intact cells, and their interaction was direct. The binding site of PR61β on Axin was different from those of GSK-3β, β-catenin, APC, and Dvl. Although PR61β did not affect the stability of β-catenin, it inhibited Dvl- and β-catenin-dependent T cell factor activation in mammalian cells. Moreover, it suppressed β-catenin-induced axis formation and expression of siamois, a Wnt target gene, in Xenopus embryos, suggesting that PR61β acts either at the level of β-catenin or downstream of it. Taken together with the previous observations that PR61 interacts with APC and functions upstream of β-catenin, these results demonstrate that PR61 regulates the Wnt signaling pathway at various steps.

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The abbreviations used are: GSK-3β, glycogen synthase kinase-3β; APC, adenosomatous polyposis coli; Tcf, T cell factor; PP2A, protein phosphatase 2A; PP2A(CA), PP2A that consists of the C and A subunits; Axin, rat Axin; MBP, maltose-binding protein; GST, glutathione S-transferase; HA, hemagglutinin; Xβ-globin, Xenopus globin; CHAPS, 3-(3-cholamidopropyl)dimethylammonio) propane sulfonic acid; DAI, dorso-anterior index; RT-PCR, reverse transcription-polymerase chain reaction.

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lates Axin and APC (16). It has been recently reported that the B56 subunit of PP2A interacts with APC and that its expression reduces the level of β-catenin and inhibits the transcription of β-catenin target gene (43). However, the mode of action of B56/PR61 in the Wnt signaling pathway is not known. In this study, we demonstrate that PR61 binds to Axin in intact cells and in vitro. We also show that PR61 does not affect the stability of β-catenin but that it inhibits Dvl- and β-catenin-induced Tcf activation in mammalian cells. Further, we demonstrate that PR61 induces ventralization and suppresses β-catenin-dependent axis duplication in Xenopus embryos, suggesting that PR61 functions either at the level of β-catenin or further downstream. Taken together with the previous observations that PR61 upstream of β-catenin (43), these results suggest that PR61 negatively regulates the Wnt signaling pathway at various steps. Because PR61 and B56 denote the same molecule (41), we use the name of “PR61” in this study.

**EXPERIMENTAL PROCEDURES**

**Materials and Chemicals**—pCMV-Flag/PP2A(C), pcDNA3/Flag-rAxin, pcDNAH/hTcf-4E, and pTOPFLASH, and pUC/EF-1α-catenin RNA were supplied by Dr. Y. Yonezawa (Kobe, Japan), R. Miyazono (Tokyo University, Tokyo, Japan), H. Clevers (University Medical Center, Utrecht, The Netherlands), and A. Nagafuchi (Kumamoto University, Kumamoto, Japan), respectively. β-Catenin RNA is a β-catenin mutant in which serine and threonine residues of the GSK-3β phosphorylation sites are changed to alanine (6). The anti-MBP and anti-GST antibodies were kindly supplied by Dr. M. Nakata (Sumitomo Electronics, Yokohama, Japan). Wnt-3a conditioned medium was prepared as described (44). To generate the anti-phosphorylated Axin antibody, three phosphorylated peptides of rAxin, PA322 (Cys-Leu-Ala-Pro-Ala-Ala), were synthesized, and were purified from bovine serum albumin (50 μg) for 30 min at 37 °C. After the incubation, the precipitates were probed with the anti-MBP antibody, and the immunoprecipitates were subjected to protein A subunits were purified from human erythrocytes (38). MBP- and GST-fused proteins were purified from Escherichia coli according to the manufacturer’s instructions. The anti-Myc antibody was prepared from 9E10 cells. COS-293L or PC12 cells were stably transfected with Flag-rAxin, wild type or L/PR61 (35-mm-diameter dish) with 0.5 μg of pEF-BOS-HA/hTcf-4E, and 0.5 μg of pUC/EF-1α-catenin into the vectors. The entire PCR products were subcloned into the BegII site of pSP64T. Sense mRNA was obtained by in vitro transcription of linearized templates using SP6-mMESSAGE mMACHINE kit (Ambion, Austin, TX). Fertilized eggs were dejellied using 4.5% cysteine acid, and the indicated mRNAs were injected into dorsal or ventral blastomeres at the four-cell stage. After injection, embryos were cultured for 3 days (at stage 40–41). UV light irradiation was performed as described (18). The particular injection of the injected line was checked by DAI (49). To carry out RT-PCR, total RNAs of embryos at stage 10.5 were incubated with 10 pmol of GST-PR61 immobilized on glutathione-Sepharose 4B in 50 μl of reaction mixture (20 μM Tris/HCl, pH 7.5, 1 mM dithiothreitol, and 0.5% CHAPS) for 1 h at 4 °C. After GST-PR61 was precipitated by centrifugation, the precipitates were probed with the anti-MBP antibody, and the precipitates were subjected to protein A subunits were purified from bovine serum albumin (50 μg) for 30 min at 37 °C. After the incubation, the precipitates were probed with the anti-MBP antibody, and the immunoprecipitates were incubated with 6 units of alkaline phosphatase in 20 μl of phosphatase reaction mixture (50 μM Tris/HCl, pH 8.0, 10 mM MgCl2, and 0.1 mg/ml bovine serum albumin) for 30 min at 37 °C. After the incubation, the mixtures were probed with the anti-Myc or anti-P322 antibody. To show that the anti-P322 antibody detects the direct phosphorylation of Axin by GSK-3β, 0.2 μM pMyc-rAxin (GFP-rAxin-298–506), or pMyc-rAxin (298–506) was incubated with 50 μM GST-GSK-3β in 30 μl of kinase reaction mixture (50 μM Tris/HCl, pH 7.5, 10 mM MgCl2, 1 mM dithiothreitol, and 50 μM ATP) for 30 min at 37 °C. After the incubation, the mixtures were probed with the anti-P322 antibody.

β-Catenin Accumulation and Tcf Assays—To observe the effect of PP2A on β-catenin, wild type or L/PR61 (35-mm-diameter dish) were deprived of serum for 6 h, then treated with Wnt-3a conditioned medium for 12 h. The lysates were probed with the anti-β-catenin antibody. To examine whether PR61 affects the Wnt-3a-dependent Tcf-4 activation, wild type L cells or L/PR61 cells (35-mm-diameter dish) were transfected with 0.5 μg of pTOPFLASH, 0.1 μg of pEBOSS-HA/hTcf-4E, and 0.5 μg of pME18S/Flag-PP2A(C) in 293 or PC12 cells. The cells were purchased for commercial sources (Lexington, KY). Other materials were obtained from commercial sources.

**Plasmid Construction**—Standard recombinant DNA techniques were used to construct the following plasmids, pBTMT16HA/Axil, pEF-BOS-HA/PR61β, pEGX-KG/PR61β, pSP44T-Myc/PR61β, pEF-BOS-HA/PR61γ, pBJ-J/PR61β-3HA, pEF-BOS-Myc/rAxin RNA85, pMAL-c2r/Axin (229–506), and pMAL-c2r/Axin (229–506) (7). The truncated constructs were used in these plasmids for in vitro transcription and analysis. The sense RNAs were transcribed linearized templates using SP6-mMESSAGE mMACHINE kit (Ambion, Austin, TX). Fertilized eggs were dejellied using 4.5% cysteine acid, and the indicated mRNAs were injected into dorsal or ventral blastomeres at the four-cell stage. After injection, embryos were cultured for 3 days (at stage 40–41). UV light irradiation was performed as described (18). The specificity of the injected line was checked by DAI (49). To carry out RT-PCR, total RNAs of embryos at stage 10.5 were isolated. Oligo(dT) primed cDNAs were synthesized using 5 μg of total RNA from 10 embryos. PCR analyses (35 cycles) were performed with ExTag DNA polymerase (Takara). Primers for PCR are: EF-1α, 5′-CAG ATT GGT GGT GGA TAT GC-3′ and 5′-ACT GCC TGT ATG ACT CCT ACG AT-3′; staisos, 5′-AAG ATA ACT GCC ATT CCT GAG C-3′ and 5′-GAT AGG CCT GTO TGT TAA GGG 5′.
PP2A and Wnt Signaling

RESULTS

Interaction of PR61 with Axin—To identify proteins that physically interact with Axin (10), an Axin homolog, we conducted a mouse brain cdNA library screening by the yeast two-hybrid method. From \(1.7 \times 10^5\) initial transformants, two clones, PR61\(\beta\) and PR61\(\gamma\), were found to confer both the His\(^+\) and LacZ\(^-\) phenotypes on L40 expressing Axin. Both proteins are known to be regulatory subunits of PP2A and to regulate its phosphatase activity and subcellular localization (41, 42). In the two-hybrid assay, Axin also interacted with PR61\(\beta\) and PR61\(\gamma\) (data not shown), suggesting that PR61 might regulate the functions of both Axin and Axil. Because Axin has been well characterized (8, 9), we used Axin to examine this possibility. Various deletion mutants of rAxin in this study are shown in Fig. 1.

First we examined whether PR61 forms a complex with Axin in intact cells. Myc-rAxin was co-expressed with HA-PR61\(\beta\) or HA-PR61\(\gamma\) in COS cells (Fig. 2A, lanes 1–5). When the lysates co-expressing Myc-rAxin with HA-PR61\(\beta\) were immunoprecipitated with the anti-Myc antibody, HA-PR61\(\beta\) was detected in the Myc-rAxin immune complex (Fig. 2A, lane 8). Similarly, when the lysates co-expressing Myc-rAxin with HA-PR61\(\gamma\) were immunoprecipitated with the anti-Myc antibody, HA-PR61\(\gamma\) was detected in the Myc-rAxin immune complex (Fig. 2A, lane 9). When the lysates expressing either HA-PR61\(\beta\) or HA-PR61\(\gamma\) alone were immunoprecipitated with the anti-Myc antibody, neither HA-PR61\(\beta\) nor HA-PR61\(\gamma\) was detected in the immunoprecipitates (Fig. 2A, lanes 6 and 7). Further, HA-PR61\(\alpha\) also formed a complex with Myc-rAxin in L cells (data not shown). To determine which region of rAxin forms a complex with PR61\(\beta\) in intact cells, various deletion mutants of Myc-rAxin were co-expressed with HA-PR61\(\beta\) in COS cells (Fig. 2B, lanes 1–7). When the lysates expressing Myc-rAxin mutants were immunoprecipitated with the anti-Myc antibody, HA-PR61\(\beta\) was coprecipitated with Myc-rAxin (full-length), Myc-rAxin\(\Delta\text{DIX}\), and Myc-rAxin-(1–529) but not with Myc-rAxin-(1–229), Myc-rAxin-(298–506), or Myc-rAxin-(713–832) (Fig. 2B, lanes 8–13). These results indicate that Axin forms a complex with PR61 in intact cells and that the N-terminal region of Axin (1–529 amino acids) containing the RGS (regulators of the G protein signaling) domain and the GSK-3\(\beta\)- and \(\beta\)-catenin-binding sites is necessary for the interaction with PR61, but none of these individual sites is sufficient.

To examine whether the interaction of Axin with PR61 is direct, MBP-rAxin, its deletion mutants, and GST-PR61\(\beta\) were purified from E. coli (Fig. 2C, lanes 1–7). MBP-rAxin (full-length) bound to GST-PR61\(\beta\) (Fig. 2C, lane 8). Among various deletion mutants of MBP-rAxin, only MBP-rAxin-(1–529) interacted with GST-PR61\(\beta\), but MBP-rAxin-(1–229), MBP-rAxin-(229–506), MBP-rAxin-(508–832), and MBP did not bind to GST-PR61\(\beta\). These results indicate that Axin interacts with PR61 via the RGS domain and the GSK-3\(\beta\)- and \(\beta\)-catenin-binding sites.

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This trimer containing GST-PR61 by GSK-3 generated the antibodies that recognize Axin phosphorylated phosphorylation level of Axin in intact cells. To this end, we brAxin is a phosphorylation site of GSK-3 MBP were precipitated by centrifugation. The precipitates were probed (lanes 4–6) were incubated with 7.5 pmol of either MBP-rAxin (lanes 1–3) and MBP (lanes 3 and 4) immobilized on amylose resin, and then MBP-rAxin and MBP were precipitated by centrifugation. The precipitates were probed with the anti-GST and anti-PP2A(C) antibodies. The results shown are representative of three independent experiments.

(Fig. 2C, lanes 9–13). These results indicate that rAxin-(1–529) directly interacts with PR61β, consistent with the observations in intact cells. Because PR61 is a subunit of PP2A (41, 42), PP2A(C) consisting of C and A subunits binds to Axin (16), and PR61β binds to Axin, we next examined whether the trimer of PP2A associates with Axin. When COS cell lysates were incubated with MBP-rAxin, endogenous PP2A(C) was coprecipitated with MBP-rAxin (Fig. 3A). Expression of HA-PR61β enhanced the complex formation of PP2A(C) with MBP-rAxin (Fig. 3A). As a large excess of MBP-rAxin was used in this experiment, these results indicate that the Axin complex is not saturated with PP2A and that PR61 can indeed recruit extra PP2A(CA). To make the trimer of PP2A in vitro, free PP2A(CA) was removed after GST-PR61β had been incubated with purified PP2A(CA). This trimer containing GST-PR61β and PP2A(CA) bound to MBP-rAxin (Fig. 3B). Taken together, these results demonstrate that the trimer of PP2A can form a complex with Axin.

Effect of PR61 on Dephosphorylation of Axin by PP2A—As we already showed that PP2A(CA) dephosphorylates APC and Axin in vitro (16), we examined whether PR61 affects the phosphorylation level of Axin in intact cells. To this end, we generated the antibodies that recognize Axin phosphorylated by GSK-3β. Because at least one of Ser322, Ser326, or Ser330 of rAxin is a phosphorylation site of GSK-3β in vitro (7), rabbit polyclonal antibodies that detect the phosphorylation of these serine residues were generated. Among the anti-P322, anti-P326, and anti-P330 antibodies, only the anti-P322 antibody detected Myc-rAxin when it was expressed in COS cells (Fig. 4A, lanes 1–4). When Myc-rAxin was immunoprecipitated from L/Axin cells with the anti-Myc antibody, the anti-P322 anti-body detected Myc-rAxin (Fig. 4A, lane 5). This band disappeared by the treatment with alkaline phosphatase (Fig. 4A, lane 6). The anti-P322 antibody did not recognize two Axin mutants, rAxinSA and rAxinD (GSK3′) (lane 4), when they were expressed in L cells (Fig. 4A, lanes 7–9). rAxinSA is an Axin mutant in which all of Ser322, Ser326, and Ser330 of rAxin are substituted with alanine. This Axin mutant was degraded more rapidly than the wild type because the phosphorylation of Axin by GSK-3β is important for its stability (21). rAxinD(GSK3′) is an Axin mutant in which its GSK-3′-binding site is deleted. This mutant functions dominant negatively in the Wnt signaling pathway, thereby accumulating β-catenin (17). Treatment of L
cells with okadaic acid enhanced the detection of endogenous Axin by this antibody (Fig. 4B). To examine whether the anti-P322 antibody detects direct phosphorylation of Axin by GSK-3β, purified MBP-rAxin was incubated with GST-GSK-3β. This antibody detected MBP-rAxin incubated with GST-GSK-3β in the presence of ATP but not in the absence of ATP (Fig. 4C, lanes 1 and 2). Furthermore, the anti-P322 antibody recognized MBP-rAxin-(298–506) but not MBP-rAxin-(298–506)S322A, in which Ser322 is mutated to alanine (7), when these rAxin mutants were incubated with GST-GSK-3β and ATP (Fig. 4C, lanes 3 and 4). From these results, we conclude that Ser322 of rAxin is indeed phosphorylated by GSK-3β and that the anti-P322 antibody is useful for detecting the phosphorylation of Axin by GSK-3β.

Using the anti-P322 antibody, the effect of PR61 on the phosphorylation of Axin in intact cells was examined. We previously demonstrated that dephosphorylation of Axin induces its down-regulation and that PP2A dephosphorylates Axin (16, 21). Expression of the catalytic subunit decreased the protein level of Axin (Fig. 4D, lanes 2 and 3). Consistent with this observation, the phosphorylation of Axin was also decreased (Fig. 4D, lanes 7 and 8). However, expression of PR61β did not affect the phosphorylation state and the stability of Axin in the presence or absence of PP2A(C) (Fig. 4D, lanes 4, 5, 9, and 10). The complex formation of PP2A with Axin through PR61β may not affect the dephosphorylation of Ser322 of rAxin.

Effect of PR61 on β-Catenin Signaling—It was shown that B56β (PR61β) down-regulates β-catenin in 293 cells and inhibits Xwnt-8-induced Xenopus siamois mRNA expression (43). To analyze the effect of PR61β on the Wnt-dependent stabilization of β-catenin, we made L cells stably expressing HA-PR61β (L/PR61β cells). We have previously shown that Wnt-3a-conditioned medium induces the accumulation of β-catenin and the activation of Tcf in L cells and that expression of Axin inhibits these Wnt-3a-dependent responses (20). Expression of PR61β in L cells did not affect the Wnt-3a-induced accumulation of β-catenin (Fig. 5A). However, Wnt-3a-dependent Tcf activation was reduced in L/PR61β cells (Fig. 5B). These results suggest that PR61β inhibits the Wnt signaling pathway through a mechanism independent of the regulation of β-catenin stability.

To clarify this possibility, we examined whether PR61 regulates Dvl- and β-catenin-dependent Tcf activation. Expression of PP2A(C) or PR61β in PC12 cells did not affect basal Tcf-4 activity (Fig. 6A, lanes 3 and 5). Dvl activated Tcf-4 in PC12 cells (Fig. 6A, lane 2). PP2A(C) enhanced Dvl-dependent Tcf activation, whereas PR61β suppressed it (Fig. 6A, lanes 4 and 6). Further, Dvl- or β-catenin-dependent Tcf-4 activation was suppressed by PR61β in 293 cells (Fig. 6B). These results suggest that PR61 may function either at the level of β-catenin or further downstream and that it antagonizes the functions of the catalytic subunit of PP2A.

Regulation of Axis Formation by PR61—To examine the role of PR61 in the Wnt signaling pathway, it was expressed in Xenopus embryos, and the axis formation was observed, because the Wnt signaling pathway regulates axis formation of Xenopus embryos (51). Ventral injection of PR61β mRNA into four-cell stage embryos resulted in ventralizing phenotypes such as loss of head in a dose-dependent manner (Fig. 7, A and B). Ventral injection of PR61β mRNA had no effect on the formation of axis (Fig. 7, A and B), siamois is a homeobox gene that mediates the effect of the Wnt signaling pathway on axis formation and whose expression is activated by β-catenin/Tcf (52, 53). Expression of siamois was suppressed by dorsal but
or ventrally (b and c) with the indicated amounts of Xglobin or PR61β mRNA. The phenotypes were expressed as DAI. DAI 0, completely ventralized; DAI 5, normal. C, inhibition of siamois expression by PR61. Embryos were injected ventrally (V) or dorsally (D) with the indicated amounts of Xglobin or PR61β mRNA. siamois expression in embryos was detected with RT-PCR analysis. The amounts of cDNA were standardized with EF-1α.

not ventral injection of PR61β mRNA in a dose-dependent manner (Fig. 7C). These results suggest that PR61β exhibits the ventralizing activity in Xenopus embryos. It has been shown that ventral injection of Xenopus β-catenin (Xβ-catenin) induces a secondary dorsal axis (54) (Fig. 8, A and B). Coexpression of Xβ-catenin and PR61β mRNAs in the ventral side demonstrated no secondary structure (Fig. 8, A and B). It was shown that UV light-irradiated embryos exhibit axial deficiencies (Fig. 8C, a) (48). PR61β alone did not affect this phenotype (Fig. 8C, b). Consistent with previous observations, Xβ-catenin rescued the UV-induced axial deficiencies (Fig. 8C, c). Coexpression with PR61β inhibited this activity of Xβ-catenin (Fig. 8C, d). The average DAI of the embryos is shown in Fig. 8D. β-Catenin recovered the expression level of siamois, which was inhibited by UV light irradiation. PR61β inhibited the β-catenin-induced expression of siamois (Fig. 8E). Therefore, it seems that PR61β inhibits the Wnt signaling pathway independently of regulation of the stability of β-catenin.

**DISCUSSION**

In this report, we show that the regulatory subunit (PR61) of PP2A directly binds to Axin and that it inhibits β-catenin-dependent Tcf activation and Xenopus axis duplication without degradation of β-catenin. These results suggest that PR61 suppresses the Wnt signaling by functioning either at the level of β-catenin or its downstream targets. PP2A consists of three subunits, a catalytic C subunit, a constant regulatory A subunit, and another regulatory B subunit (41, 42). PP2A can exist in the form of either CA dimeric core or heterotrimeric CAB holoenzyme. Free C subunit is not found in the cells, but other dimeric forms, consisting of C and Tap42/PR55, are present (55). There are three structurally unrelated B families, B/PP55, B/β56/PR61, and B/PR72/PR59. Each family has several related proteins and isoforms with tissue-specific expression (39, 40). Association of different regulatory B subunits with PP2A(CA) can result in altered substrate specificity, catalytic activity, and subcellular localization (41, 42). The PR61 family contains at least five distinct gene products denoted α, β, γ, δ, and ε and splice variants, all highly homologous with each other. The central region of the molecules is conserved among different isoforms, whereas the N- and C-terminal regions are divergent. As PR61α, -γ, and -δ form a complex with Axin, it is possible that the other isoforms will bind as well. We have previously shown that PP2A(CA) binds to Axin (16). Further-
more, our results demonstrate that the trimer of PP2A can indeed associate with Axin. However, we do not know whether this is via PR61, via PP2A(AC), or via both.

PP2A(AC) dephosphorylates Axin and APC in vitro (16). Therefore, when we found that PR61 binds to Axin directly, we speculated that PR61 regulates PP2A activity thereby regulating the stability of β-catenin. However, PR61β does not affect the phosphorylation of Axin by GSK-3β in intact cells. GSK-3β efficiently phosphorylates β-catenin and Axin in the Axin complex (7). Because the phosphorylation state of Axin is thought to reflect that of β-catenin by GSK-3β, it was speculated that PR61 is not involved in the stability of β-catenin. Indeed, expression of PR61β did not affect Wnt-3a-dependent β-catenin accumulation in L cells (Fig. 5A). These results are not consistent with the observations that B56 regulatory domain (PR61β) down-regulates β-catenin in 293 cells (43). Although the reasons for the discrepancy are not known, it might be because of the differences of the assay conditions. We have examined the stability of endogenous β-catenin, whereas ectopically expressed β-catenin was tested in another report (43). Although we cannot exclude the possibility that other PR61s regulate PP2A activity for the dephosphorylation in the Axin complex, the physiological significance of the binding of PR61 to Axin is not known at present. Because PR61 also interacts with APC (43), it may bridge APC and Axin, resulting in the making of a more stable complex. Alternatively, PR61 isoforms may determine the subcellular localization of Axin.

Although PR61β did not affect the stability of β-catenin, we found that it suppresses Wnt-3a-, Dvl-1-, and β-catenin-dependent Tcf-4 activation. The C subunit of PP2A enhanced Dvl-1-dependent Tcf-4 activation. Therefore, the dephosphorylation activity of PP2A may be involved in the regulation of transcriptional activity of Tcf-4. Moreover, PR61β induced ventralization, and it inhibited β-catenin-dependent axis duplication in Xenopus embryos. These results suggest that PR61β acts as a negative regulator of Wnt signaling by functioning either at the level of β-catenin or further downstream. During the preparation of this manuscript, it was shown that B′β (PR61ε) does not affect β-catenin stability in Xenopus embryos but that Xenopus disheveled- and β-catenin-dependent expression of siamois is enhanced by the catalytic subunit of PP2A (56). Furthermore, disheveled-induced axis duplication is enhanced and inhibited by the catalytic subunit and B′ε, respectively (56). These results suggest that PP2A(C) and PR61ε regulate the Wnt signaling pathway in Xenopus embryos and are consistent with our observations.

Although the detailed mechanism of how PP2A causes an altered level of Tcf activation in the Wnt signaling pathway remains to be elucidated, the regulation may occur via subcellular localization of β-catenin and/or the complex formation of β-catenin, Tcf, and DNA. Tumor suppressor gene product APC, which binds to β-catenin directly, induces nuclear export of β-catenin (57, 58). APC is phosphorylated by GSK-3β, and the phosphorylation enhances the binding of APC to β-catenin (59). It is intriguing to speculate that PP2A dephosphorylates APC, thereby inhibiting nuclear export of β-catenin. This is at least one mechanism whereby the C and PR61β subunits of PP2A would regulate the Wnt signaling pathway via a mechanism independent of β-catenin stabilization. The complex formation of β-catenin, Tcf, and DNA is regulated by several proteins including Groucho (60), CBP (cAMP-response element-binding protein-binding protein) (61), CBP (C-terminal binding protein family of transcriptional corepressors) (62), NLK (NF-kB essential modulator like kinase) (63), ICAT (64), Pontin52 and Reptin52 (65), and Duplin (66). Among these proteins, NLK directly binds to and phosphorylates Tcf, and the phosphorylation of Tcf inhibits the complex formation of β-catenin, Tcf, and DNA (63). PP2A may act on Tcf and inhibit the phosphorylation of Tcf by NLK, thereby enhancing Tcf-dependent transcriptional activation.

We have generated the antibody that recognizes phosphorylated Ser1222 of rAxin in this study. Previous observations have demonstrated that Ser1222 is one of the possible phosphorylation sites by GSK-3β in vitro (7). The present results clearly show that Ser1222 of rAxin is a target of GSK-3β in intact cells, although it has been demonstrated that Thr456 and Ser490 of Axin are also physiological phosphorylation sites by GSK-3β (67). We have also reported that phosphorylation by GSK-3β stabilizes Axin in contrast to β-catenin (21). Consistent with this observation, the protein levels of Axin and Axin (GSK-3β), which are not phosphorylated by GSK-3β, are reduced in comparison with that of wild-type Axin (Fig. 4A). Therefore, this antibody is useful for examining the relationship between the phosphorylation of Axin and its stability.

In summary, we showed that PR61 is a new binding partner of Axin and that it negatively regulates the Wnt signaling pathway. Although the mode of the inhibitory action of PR61 in this signaling pathway is not known, the results suggest that PR61 acts at the level of β-catenin or downstream of it. Further studies are necessary for understanding how PP2A regulates the Wnt signaling pathway.

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