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A GSK3β Phosphorylation Site in Axin Modulates Interaction with β-Catenin and Tcf-Mediated Gene Expression

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Upon binding of a Wnt to its receptor, GSK3β is inhibited through an unknown mechanism involving Dishevelled (Dsh), resulting in the dephosphorylation and stabilization of β-catenin, which translocates to the nucleus and interacts with Lef/Tcf transcription factors to activate target gene expression. Axin is a scaffold protein which binds β-catenin and GSK3β (as well as several other proteins) and thus promotes the phosphorylation of β-catenin. Here we report that Axin is phosphorylated on Ser and Thr residues in several regions *in vivo,* while only one region (amino acids 600–672) is efficiently phosphorylated by GSK3β *in vitro.* Site-directed mutagenesis, together with *in vitro* and *in vivo* phosphorylation assays, demonstrates that Axin residues T609 and S614 are physiological GSK3β targets. Substitutions for one or more of these residues, which lie within a β-catenin binding site, reduce the ability of Axin to modulate Wnt-induced signaling in a Lef/Tcf reporter assay. These amino acid substitutions also reduce the binding between Axin and β-catenin. We propose a model in which inhibition of GSK3β activity upon Wnt signaling leads to the dephosphorylation of GSK3β sites in Axin, resulting in the release of β-catenin from the phosphorylation complex. © 1999 Academic Press

The regulation of cytoplasmic β-catenin levels is believed to be a key step in the Wnt signal transduction pathway (1–4). In the absence of a Wnt signal, β-catenin is phosphorylated by GSK3β, leading to its degradation through the ubiquitination/proteasome pathway (5, 6). Upon binding of a Wnt to its receptor, the activity of glycogen synthase kinase 3β (GSK3β) is inhibited through an unknown mechanism involving Dishevelled (Dsh in *Drosophila;* Dvl in mammals), leading to the dephosphorylation of β-catenin (7). Dephosphorylated β-catenin is stabilized and translocated into the nucleus, where it activates Lef/Tcf-mediated gene expression (8–10). Deregulation of the β-catenin level due to mutations in the *adenomatous polypopsis coli (APC)* tumor suppressor or *β-catenin* genes initiates colorectal and other forms of neoplasia (11–13).

Recently we and others showed that Axin, the product of the murine genetic locus originally called *Fused,* plays a pivotal role in the down regulation of β-catenin levels and inhibition of Wnt signal transduction (14–17). It is now well established that Axin is a component of the multi-protein complex including GSK3β, APC and β-catenin, which serves to regulate β-catenin levels (15, 16, 18–22). The region of Axin that binds to GSK3β and β-catenin is sufficient to promote the phosphorylation of the latter *in vitro* (15), although the APC binding domain of Axin is required for its ability to regulate β-catenin levels in frog embryos (22). Dsh co-localizes with Axin in co-injected frog embryos (22), in MDCK cells (23), and also binds to Axin in yeast two-hybrid assays (24), suggesting that Dsh is also part of the Axin complex. Finally, the catalytic subunit of the Ser/Thr protein phosphatase 2A ($PP2A_c$) can bind directly to Axin (25) and a PP2A regulatory subunit, B56, can bind to APC (26), suggesting that dephosphorylation by PP2A may modulate the effects of phosphorylation by GSK3β (or other Ser/Thr kinases).

In vitro experimental data suggested that Axin, as well as APC and β-catenin, is a substrate for GSK3β phosphorylation (15, 16, 27, 28). However, the sites of phosphorylation on Axin were not well established, and the significance of these phosphorylation events for Wnt signal transduction was unknown. Here, we report that multiple regions of Axin are phosphorylated *in vivo,* and we identify a major GSK3β phosphorylation site using site-directed mutagenesis together with *in vitro* and *in vivo* assays. A Tcf-mediated reporter assay shows that mutation of conserved Ser

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and/or Thr residues within this GSK3β phosphorylation site reduces the ability of Axin to inhibit Tcf signaling. These amino acid substitutions also reduce the interaction of Axin with β-catenin, which may account for their effects. Our data suggest a model in which the inhibition of GSK3β activity upon Wnt signaling leads to dephosphorylation of Axin, thereby preventing the binding and phosphorylation of β-catenin. Recently, Yamamoto *et al.* (29) reported that phosphorylation of Axin by GSK3β results in increased stability of Axin. However, our results differ in that we identify a different GSK3β phosphorylation site in Axin, and we provide evidence for a different mechanism by which the inhibition of Axin phosphorylation by GSK3β leads to β-catenin stabilization.

EXPERIMENTAL PROCEDURES

Plasmid construction. The construction of a myc-tagged form of mouse full length and deleted versions of Axin were described elsewhere (22). Site directed mutations were introduced by standard PCR techniques using *Pfu* DNA polymerase (Stratagene, La Jolla, CA). All constructs were confirmed by sequencing and detection of expected sized bands in Western blot or SDS-polyacrylamide gels stained with Coomassie brilliant blue R-250.

In vitro GSKβ and CKII kinase assay. His · S tagged Axin fusion proteins were mixed with GSK3β (2.5 units, N. E. Biolabs) or casein kinase II (250 units, N. E. Biolabs) in the buffers recommended by the manufacturer, supplemented with [γ-^{32}P]ATP (10 μCi/tube) and incubated for 15 min at 30°C.

Transient transfection and in vivo labeling. 293 cells were cultured and transfected as described previously (22). The day after DNA addition, the medium was replaced with DMEM (no phosphate) containing 0.5% fetal bovine serum and 1 mCi/ml [^{32}P]orthophosphate and incubated for 4 h at 37°C. The cells were then lysed as described elsewhere (22).

Luciferase assay. To measure β-catenin/Tcf signaling we used pTOPFLASH, a plasmid containing three copies of an optimal Tcf motif upstream of fly luciferase coding sequences (11). The expression derived from pFOPFLASH, which has three copies of a mutated Tcf motif upstream of fly luciferase, was too low, making it difficult to determine the pTOPFLASH/pFOPFLASH ratio. Therefore we used pRL-TK-*Renilla* luciferase instead, and the ratio between fly and *Renilla* luciferase was measured by Dual-Luciferase reporter assay system (Promega Corp., Madison, WI) according to the manufacturer's protocol.

Direct binding assay. The direct binding assay was performed as described previously (22).

RESULTS

Immunoprecipitation of metabolically labeled Axin and subsequent phosphoamino acid analysis showed that Axin is a phosphoprotein and that phosphorylation occurs on Ser and Thr but not Tyr residues (Fig. 1B and data not shown). Consistent with this observation, phosphotyrosine-specific antibodies do not recognize immunoprecipitated Axin (data not shown). To narrow down the phosphorylated region of Axin, several partially deleted forms of Axin (Fig. 1A) were

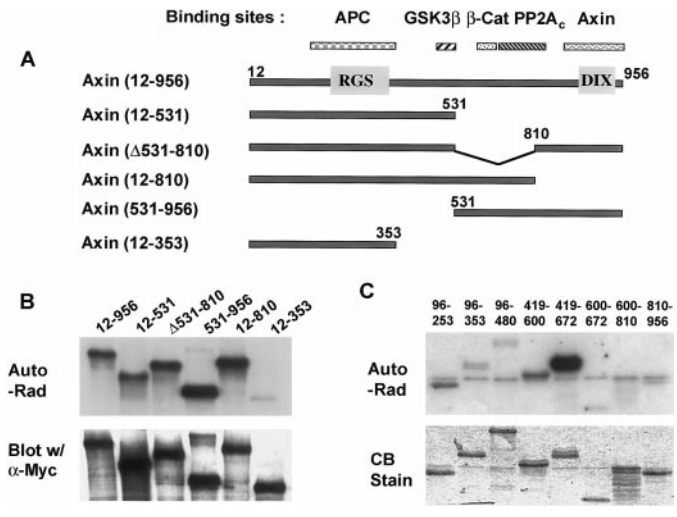

FIG. 1. *In vivo* and *in vitro* phosphorylation of Axin. (A) A diagram of myc-tagged full length Axin and other deleted versions of Axin which were transfected into 293 cells for *in vivo* labeling with $^{32}PO_4$. The binding regions for APC, GSK3β, β-catenin, PP2A$_c$ and Axin itself, and the regions of Axin homologous to the RGS domain and to Dsh (DIX domain) are indicated. (B) *In vivo* labeled myc-tagged Axins were immunoprecipitated and their expression was detected by western blot (bottom). The same blot was used for the detection of phosphorylation by autoradiography (top). (C) *In vitro* phosphorylation assay. Coomassie blue staining indicates the level of expression of S protein-tagged fusion proteins (bottom), while autoradiography of the same gel (top) shows strong phosphorylation only of Axin(419-672).

expressed in 293 cells and labeled with [^{32}P]orthophosphate. All forms besides Axin(12-353) were strongly phosphorylated *in vivo* (Fig. 1B). Since Axin(12-531) and Axin(531-956) were phosphorylated, we concluded that regions 354-531 and 531-956 of Axin both contain phosphorylation sites. A comparison of two-dimensional peptide mapping and HPLC analysis (data not shown) between full length Axin, Axin(12-810) and Axin(Δ531-810) indicated that two separate regions C-terminal to residue 531 (531-810 and 810-956) were phosphorylated *in vivo.*

We next examined which regions of Axin could be phosphorylated by GSK3β *in vitro.* In kinase assays with GSK3β and several small Axin S-tagged fusion proteins, of the fragments we tested, only Axin(419-672) was highly phosphorylated (Fig. 1C). Since Axin(419-600) was not phosphorylated above background levels, we concluded that GSK3β phosphorylates Axin in the 600-672 region. Furthermore, since neither Axin(600-672) nor Axin(600-810) was phosphorylated, it appears that the GSK3β binding region of Axin (500-550) is also necessary for GSK3β to phosphorylate Axin at a more C-terminal site. Axin(422-810), which was also strongly phosphorylated by GSK3β, was not phosphorylated by casein kinase II (CKII), even though we used 100-fold more units of CKII than GSK3β (data not shown). Phosphoamino

FIG. 2. Substitution of alanine for T609, S614 and S624 or isoleucine for S621 reduces *in vitro* phosphorylation by GSK3β. (A) Sequence alignment of Axin from several different species and murine Conductin (Axil/AXIN2) displaying conservation of potential GSK3β phosphorylation sites. (B) *In vitro* GSK3β kinase assay with S-protein tagged Axin(419-672) containing the indicated site directed mutations. Top, autoradiograph. Middle and bottom, Coomassie blue staining of gels before and after phosphorylation by GSK3β shows a mobility shift for some of the proteins.

acid analysis showed that Axin(419-672) was phosphorylated by GSK3β on both Ser and Thr residues (data not shown). Our results are consistent with those of Ikeda *et al.* (15), who observed strong phosphorylation by GSK3β of a rat Axin fragment corresponding to mouse Axin 422-630, and together these results suggested that the major site is between 600-630. However, our data are inconsistent with their conclusion that Ser residues corresponding to S446/S450/S454 are GSK3β phosphorylation sites (see Discussion).

A comparison of the mouse Axin 600-630 region with the corresponding sequences of Axin (14, 15, 30) and the related protein conductin/Axil/AXIN2 (31–33) showed a highly conserved sequence containing potential GSK3β phosphorylation sites (S/T-X_N-S/T; Refs. 34, 35) (Fig. 2A). GSK3β phosphorylation sites tend to contain one or more nearby proline residues, and often contain a Ser/Thr residue C-terminal to the GSK3β site which is first phosphorylated by another kinase. The number of amino acids between two phosphorylated Ser/Thr residues is usually 2 to 5 but in some case 12 or 26 (35). To examine the significance of this potential GSK3β site, we introduced single or combined amino acid substitutions of Ala or Ile for Thr609, Ser614, Ser621, and Ser624, and first tested them in an *in vitro* GSK3β kinase assay. Phosphorylation was detected both by incorporation of ^{32}P (Fig. 2B, top), and by the reduced mobility of phosphorylated forms of Axin(419-672) on Coomassie blue stained gels (Fig. 2B, middle and bottom). Wild type Axin(419-672) was strongly labeled, and displayed two bands of reduced mobility presumably representing phosphorylated forms (Fig. 2B, arrows in bottom). The S624A mutant appeared identical to wild type. T609A and S614A each caused somewhat lower labeling by GSK3β, compared to wild type or S624A, and eliminated both of the reduced-mobility bands (Fig. 2B, bottom). Phosphorylation on Thr as well as Ser residues in phosphoamino acid analysis with wild type Axin(419-672) (data not shown) also supported the conclusion that T609 is a GSK3β phosphorylation site. S621I displayed only one of the two reduced mobility bands, indicating that it also affected the pattern of phosphorylation. The double mutation S614A/S621I and the triple mutation T609A/S614A/S621I strongly reduced the ^{32}P-labeling of Axin(419-672), and eliminated both reduced-mobility bands. Overall, the data indicate that T609, S614 and S621 are *in vitro* GSK3β phosphorylation targets.

We next tested whether these are physiological phosphorylation sites by *in vivo* labeling in 293 cells (Fig. 3). When introduced into full length (FL) Axin, the triple mutation T609A/S614A/S621I caused only a slight reduction in overall phosphorylation (Fig. 3A). However, this is not surprising since Axin appears to have two additional regions that are phosphorylated *in vivo* (Fig. 1 and unpublished data), most likely by other kinases. However, when the same triple mutation was introduced into Axin(497-672), a fragment including the binding sites for GSK3β and β-catenin (20, 22), it greatly reduced phosphorylation *in vivo* (Fig. 3A), suggesting that T609, S614 and S621 (or a subset of these) represent physiological GSK3β phosphorylation targets. Despite its effects on phosphorylation, this triple mutation did not visibly alter the total level of FL-Axin or Axin(497-672) in transfected 293 cells (Fig. 3A, left). Interestingly, wild type Axin(497-672) showed a weak band migrating more slowly than the major band in both western blot and autoradiograph (Fig. 3A). When Axin(497-672) containing the triple mutation (T609A/S614A/S621I) was examined, the level of the major band detected by Western blot was unchanged, but the phosphorylation of the major band was much reduced (autoradiograph), and the shifted band was eliminated in both Western blot and autoradiograph (Fig. 3A).

We suspected that the shifted band was due to *in vivo* phosphorylation of Axin by GSK3β in the 609/614/621 region. To test this possibility, we treated Axin(497-672)-transfected 293 cells with LiCl, a potent GSK3β inhibitor (36–38), or with NaCl as a negative control. The specific GSK3β inhibitor LiCl clearly eliminated the shifted band in western blot and autoradiograph (Fig. 3B), suggesting that this band is due to GSK3β phosphorylation.

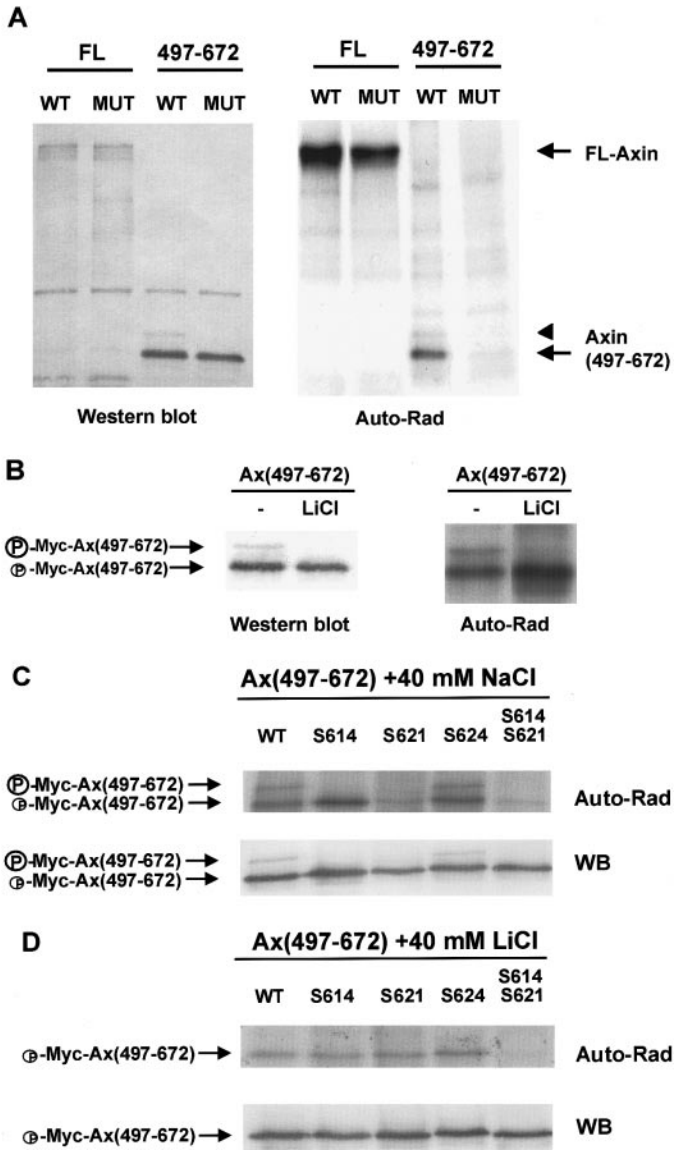

FIG. 3. Reduction of phosphorylation by site-directed mutations in potential GSK3β phosphorylation sites and the specific GSK3β inhibitor LiCl *in vivo*. (A) Wild type or triple mutant (T609A/S614A/S621I) forms of myc-tagged full-length Axin or Axin(497-672) were labeled *in vivo*, immunoprecipitated and analyzed by western blot to detect their expression and autoradiography to detect phosphorylation. The arrowhead shows a shifted band apparently caused by phosphorylation of Axin(479-672). (B) Myc-tagged Axin(497-672) was transfected into 293 cells and *in vivo* labeled. To inhibit phosphorylation by GSK3β, cells were treated with 20 mM LiCl for 2 h before labeling and 4 h during incubation with $^{32}PO_4$. (C) Several mutant forms of myc-tagged Axin(497-672) were labeled *in vivo* (in the presence of 40 mM NaCl as a control for LiCl) and immunoprecipitated with anti-myc antibody. Immunoprecipitated Axins were detected by autoradiography and western blot. (D) To inhibit GSK3β activity 40 mM LiCl was added to cells for 2 h before labeling and 4 h while incubating with $^{32}PO_4$.

Unlike the shifted band, *in vivo* phosphorylation of the major Axin(497-672) band was not visibly reduced by LiCl (Fig. 3B). Since the phosphorylation of this major band was clearly reduced by the triple mutation T609A/S614A/S621I (Fig. 3A), we hypothesized that this band represents Axin phosphorylated at S621 by a different, LiCl-insensitive kinase, while the shifted band is additionally phosphorylated by GSK3β at T609 and/or S614. To test this hypothesis, 293 cells were transfected with different mutant forms of Axin(497-672) and labeled *in vivo* with [^{32}P]orthophosphate. Consistent with our hypothesis, the S614A mutant showed strong phosphorylation of the major band but absence of the shifted band, while S621I and S614A/S621I showed a clear reduction of phosphorylation on the major band as well as elimination of the shifted band (Fig. 3C). The weak residual phosphorylation of those mutants might either be due to alternative phosphorylation by the same unknown kinase on S624 (in S621I-containing mutants) or weak phosphorylation by GSK3β without pre-phosphorylation by the other kinase on S621. WT and S624A mutant Axin(497-672) had the same shifted band, which disappeared with LiCl treatment (compare Figs. 3C and 3D). Mutants containing S614A (S614A and S614A/S621I) showed no shifted band. In summary, our *in vitro* and *in vivo* phosphorylation data strongly suggest that GSK3β phosphorylates Axin on S614 (and possibly T609) while S621 is phosphorylated by another unknown kinase *in vivo*.

To examine the role of GSK3β phosphorylation in the activity of Axin, we tested the activity of several mu-

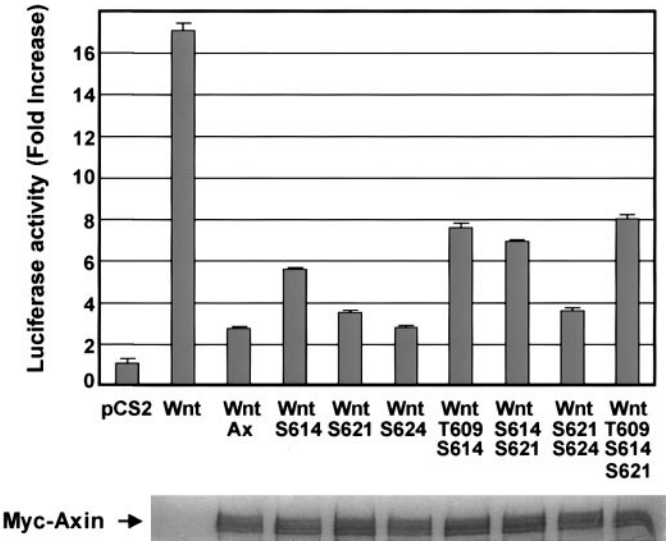

FIG. 4. Reduction of Axin activity in Tcf/Lef signaling by mutations in GSK3β phosphorylation sites. Wild type or site-directed mutant forms of full-length myc-Axin were co-transfected with Wnt1/5 to assess their ability to down-regulate Tcf/Lef signaling, using a luciferase reporter assay. This graph shows data from one representative experiment of multiple independent experiments. Error bars indicate standard deviation based on duplicate luciferase assays. The same lysates were probed with anti-myc, revealing equal expression of Axins (bottom).

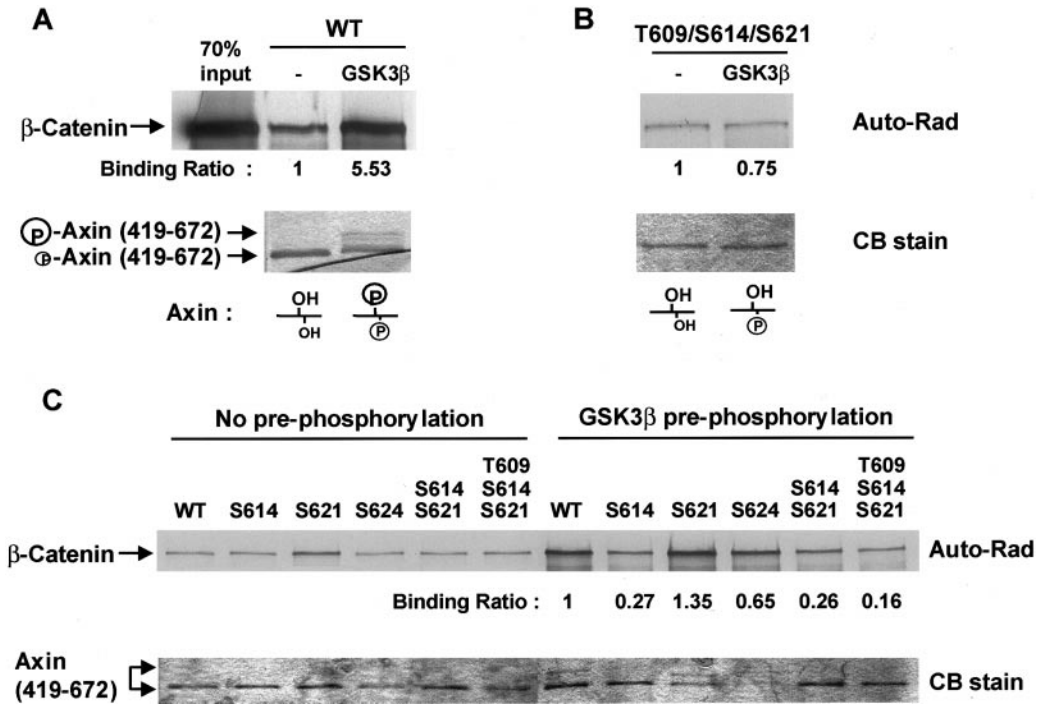

FIG. 5. Phosphorylation of Axin by GSK3β leads to increased direct binding of β-catenin *in vitro*. (A) Wild type Axin(419-672), either untreated (−) or pre-phosphorylated by GSK3β, was incubated with [^{35}S]Met-labeled β-catenin and precipitated with S-protein agarose. Top, autoradiograph. The relative amount of labeled β-catenin binding to Axin(419-672) with or without pre-phosphorylation, determined by Phosphoimager analysis, is indicated. In the first lane, 70% of the amount of β-catenin used for the direct binding assay was loaded to show the expression level of translated β-catenin ("70% input"). Middle, Coomassie blue staining shows a mobility shift in pre-phosphorylated Axin(419-672). Bottom, diagram of phosphorylation status. Small "P" stands for possible weak phosphorylation at sites other than T609/S614/S621. Larger "P" indicates phosphorylation on T609/S614/S621. (B) Same as (A) except that the triple mutant form of Axin(419-672) was used. (C) Binding of *in vitro* translated [^{35}S]Met-labeled β-catenin to Axin(419-672) containing several site-directed mutations. The first 6 lanes show binding assays with no GSK3β pre-phosphorylation and the last 6 lanes with GSK3β pre-phosphorylation of Axin(419-672). Top, autoradiograph to detect β-catenin; bottom, Coomassie blue staining to detect Axin. "Binding ratio" indicates the amount of β-catenin bound by mutant forms of Axin(419-672) relative to wild type.

tant forms of Axin in a Tcf reporter assay (11, 19, 21). Replacement of Ser/Thr residues in the phosphorylation sites provides a method to mimic a constitutively dephosphorylated form of the protein (39). According to prevailing models, the activity of GSK3β is inhibited by Wnt signaling, leading to decreased phosphorylation and accumulation of cytosolic β-catenin. This allows β-catenin to translocate to the nucleus and regulate gene expression via interaction with Lef/Tcf transcription factors (for review, see 1). Overexpression of Axin inhibits signaling through this pathway, as demonstrated by several assays, including blockage of axis formation in frog embryos (14), β-catenin downregulation (16, 22) and Tcf reporter assays (21). We reasoned that if Axin is a GSK3β substrate, it might be dephosphorylated upon exposure of cells to a Wnt signal, due to inhibition of GSK3β. Dephosphorylation of the GSK3β sites in Axin (which might be mediated by PP2A) could reduce Axin's activity, contributing to the accumulation of β-catenin. To measure Axin activity, we co-transfected HA-tagged Wnt1/5 (a highly oncogenic chimera of Wnts 1 and 5a that induces high cytoplasmic β-catenin levels) (40) together with wild type full-length Axin or several mutant forms in which one to three Ser/Thr residues were replaced with Ala or Ile. As expected, transfection of Wnt1/5 caused a large (average 14-fold) induction of Tcf-mediated luciferase activity compared to vector alone (Fig. 4), and also increased the level of β-catenin, even in total cell lysates (data not shown). Co-transfection of wild type Axin with Wnt1/5 largely inhibited the induction of luciferase activity by Wnt1/5 (Fig. 4). The S621I and S624A single mutations and the S621I/S624A double mutation had a minimal effect, if any, on the inhibitory activity of Axin in this assay. However, the S614A mutation had a small but reproducible effect, while the three double and one triple mutation that included S614A caused greater reductions in the ability of Axin to inhibit Tcf-mediated luciferase activity (Fig. 4).

We next asked why these mutated versions of Axin had a reduced ability to inhibit Wnt-induced, Tcf-mediated gene expression. The mutations did not appear to reduce the stability of Axin, as shown by the similar levels of wild type and mutant Axin in trans-

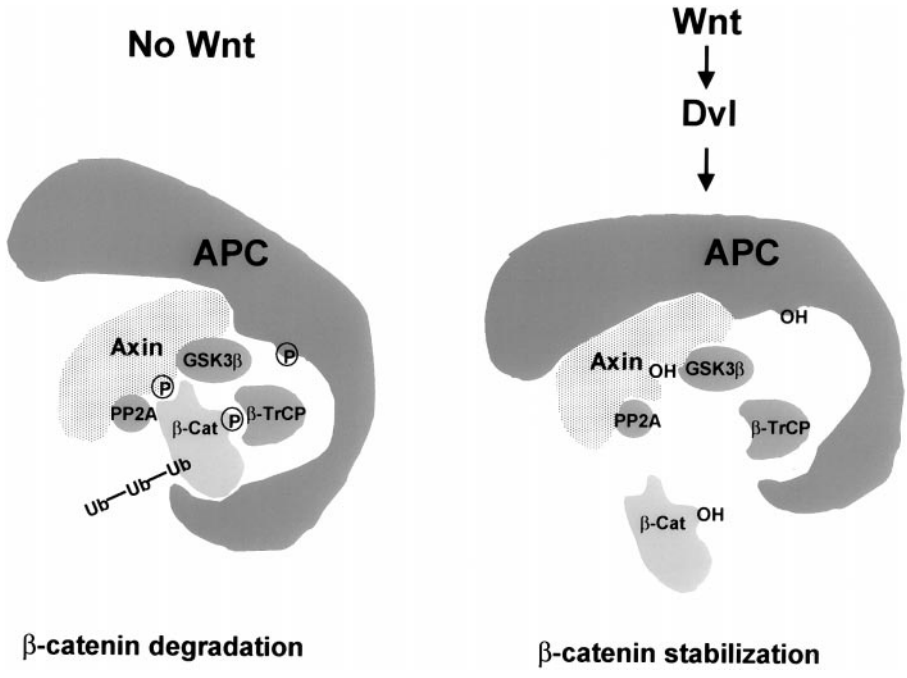

FIG. 6. A model for the role of Axin phosphorylation in the regulation of β-catenin stability. When active (left), GSK3β phosphorylates Axin as well as APC and β-catenin. The phosphorylated form of Axin binds strongly to β-catenin and promotes the phosphorylation of β-catenin by GSK3β, leading to strong interaction with β-TrCP (6, 42–44) leading to the ubiquitination and down-regulation of β-catenin. Upon Wnt signaling (left), Dvl is somehow activated, which leads to inactivation of GSK3β through an unknown mechanism, resulting in dephosphorylation of Axin (potentially by PP2A). Dephosphorylated Axin has a reduced affinity for β-catenin and releases it from the degradation complex, promoting β-catenin stabilization and signaling.

fected cells (Figs. 3A and 4, bottom). We also detected no difference in the ability of wild type vs. mutant forms of Axin to interact with GSK3β, as assayed by co-immunoprecipitation (data not shown). Therefore, we tested whether the phosphorylation of Axin by GSK3β might affect its interaction with β-catenin. ^{35}S-Met-labeled, *in vitro* translated β-catenin was mixed with S-tagged Axin(419-672), which in some cases had been pre-phosphorylated by GSK3β, and precipitated with S-protein agarose. Pre-phosphorylation of wild type Axin(419-672) increased by 5–6 fold the amount of β-catenin bound (Fig. 5A). However, pre-phosphorylation of triple mutant Axin(419-672) by GSK3β failed to enhance its binding to β-catenin (Fig. 5B). When we compared the ability of additional mutant forms of Axin(419-672) to bind to β-catenin, without pre-phosphorylation by GSK3β they all bound equally well (Fig. 5C) (although the S621I mutant bound more β-catenin in the experiment shown, this difference was not seen in several other experiments). However, when Axin(419-672) mutants were pre-phosphorylated by GSK3β the differences in binding to β-catenin were very clear (Fig. 5C). Consistent with the effects of the mutations on the Tcf reporter assay, S621I and S624A had little or no effect on β-catenin binding, while each of the three mutants containing S614A caused a 4–6 fold reduction in β-catenin binding (Fig. 5C). Overall, our results show that mutations mimicking dephosphorylation of a GSK3β target in Axin, which lies within the β-catenin binding region (amino acids 592–616 in mouse Axin, (20)), reduce the ability of Axin to bind β-catenin, and thus promote β-catenin stabilization and signaling (Fig. 6).

DISCUSSION

The ability of the Ser/Thr kinase GSK3β to phosphorylate Axin *in vitro* (15) suggested that the phosphorylation status of Axin might be altered upon Wnt signaling, and that the resulting inhibition of GSK3β activity might lead to dephosphorylation of Axin and modulation of Axin's activity. We hypothesized that the activity of Axin would be reduced by dephosphorylation. To test that hypothesis we sought to identify the sites in Axin that are phosphorylated by GSK3β *in vitro* and *in vivo*, and to ask whether amino acid substitutions at these sites would alter Axin's activity.

Within the 600-672 region, we identified an evolutionarily conserved group of Ser/Thr residues at position 609-624, constituting a potential GSK3β site. We therefore introduced single or combined amino acid substitutions at positions T609, S614, S621, and S624, and tested them in both *in vitro* and *in vivo* phosphorylation assays. From *in vitro* and *in vivo* data we could conclude that Axin is phosphorylated at S621 by an unknown LiCl-insensitive kinase, and that, *in vivo*,

this event is required for subsequent phosphorylation on S614 (and possibly T609).

Using a Tcf-mediated luciferase assay, we showed that several forms of Axin containing the S614A amino acid substitution (either alone, or together with T609A and/or S621A) displayed a reduced ability to inhibit Wnt-mediated signaling (Fig. 4). These results imply that phosphorylation at this site is necessary for maximal activity. Interestingly, the GSK3β phosphorylation site lies within the core region for β-catenin binding (15, 20, 22). Therefore, we tested whether phosphorylation of Axin affects its ability to bind to β-catenin (Fig. 5). Pre-phosphorylation of wild type Axin(419-672) by GSK3β increased by 5–6 fold the amount of β-catenin bound. Our results contrast with those of Ikeda *et al.* (15), who did not observe any effect of *in vitro* phosphorylation of rat Axin(298-506), equivalent to mouse Axin(422-630), on binding to β-catenin. Consistent with the effects of the mutations we observed in the Tcf reporter assay, S621I and S624A had little or no effect on β-catenin binding, while each of the three mutants containing S614A caused a 4–6 fold reduction in β-catenin binding (Fig. 5C). These differences in binding to β-catenin were only observed when the wild type and mutant forms of Axin(419-672) were pre-phosphorylated by GSK3β. This strongly supports the idea that the phosphorylation of Axin at GSK3β sites within the β-catenin binding domain is essential for optimal interaction with β-catenin.

Thus, an important consequence of reduced GSK3β activity following a Wnt signal may be the dephosphorylation of Axin (possibly by PP2A), leading to a reduced efficiency of binding to β-catenin. The escape of β-catenin from phosphorylation by GSK3β and subsequent degradation would thus account for its accumulation following exposure to Wnt or other GSK3β inhibitors (Fig. 6). While mutations in the GSK3β site of Axin reduced significantly, but did not eliminate, the activity of Axin in the Tcf-luciferase activity (Fig. 4), this may be due to the residual ability of the mutant Axins to bind β-catenin (Fig. 5), together with the fact that they are overexpressed compared to endogenous levels.

Recently, Yamamoto *et al.* (29) reported that the triple mutant rat Axin S322A/S326A/S330A (corresponding to residues S446, S450 and S454 of mouse Axin) was less stable than wild type Axin, and concluded that phosphorylation of Axin at these sites by GSK3β (15) regulates its stability. As noted above, in our experiments the region of mouse Axin containing S446, S450 and S454 was not phosphorylated by GSK3β *in vitro*. Furthermore, in multiple experiments (Figs. 3, 4 and data not shown), Western blot analysis did not reveal any difference in the expression level of wild type Axin vs. forms with Ser/Thr substitutions in the GSK3β phosphorylation site (T609/S614). Our data therefore suggest an alternative model, in which the phosphorylation of Axin by GSK3β enhances Axin's ability to interact with β-catenin.

While this paper was in preparation, Willert *et al.* (41) reported similarly that Wnt-induced dephosphorylation of Axin leads to reduced binding to β-catenin. Our results complement those of Willert *et al.*, in that we used a different biochemical approach and reached similar conclusions. In addition, we identified specific GSK3β phosphorylation sites in Axin that mediate its interaction with β-catenin.

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