

# Identification of a Domain of Axin That Binds to the Serine/Threonine Protein Phosphatase 2A and a Self-binding Domain\*

(Received for publication, July 15, 1998, and in revised form, November 11, 1998)

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**Axin is a negative regulator of embryonic axis formation in vertebrates, which acts through a Wnt signal transduction pathway involving the serine/threonine kinase GSK-3 and  $\beta$ -catenin. Axin has been shown to have distinct binding sites for GSK-3 and  $\beta$ -catenin and to promote the phosphorylation of  $\beta$ -catenin and its consequent degradation. This provides an explanation for the ability of Axin to inhibit signaling through  $\beta$ -catenin. In addition, a more N-terminal region of Axin binds to adenomatous polyposis coli (APC), a tumor suppressor protein that also regulates levels of  $\beta$ -catenin. Here, we report the results of a yeast two-hybrid screen for proteins that interact with the C-terminal third of Axin, a region in which no binding sites for other proteins have previously been identified. We found that Axin can bind to the catalytic subunit of the serine/threonine protein phosphatase 2A through a domain between amino acids 632 and 836. This interaction was confirmed by *in vitro* binding studies as well as by co-immunoprecipitation of epitope-tagged proteins expressed in cultured cells. Our results suggest that protein phosphatase 2A might interact with the Axin-APC-GSK-3- $\beta$ -catenin complex, where it could modulate the effect of GSK-3 on  $\beta$ -catenin or other proteins in the complex. We also identified a region of Axin that may allow it to form dimers or multimers. Through two-hybrid and co-immunoprecipitation studies, we demonstrated that the C-terminal 100 amino acids of Axin could bind to the same region as other Axin molecules.**

Axin, the product of the mouse gene originally called *Fused* (1), has been shown to negatively regulate an early step in vertebrate embryonic axis formation, through its ability to modulate a Wnt signal transduction pathway (2). The *Fused* allele (*Axin<sup>Fu</sup>*), as well as two other spontaneous alleles, *Kinky* (*Axin<sup>Ki</sup>*) and *Knobbly* (*Axin<sup>Kb</sup>*), caused similar dominant phenotypes characterized mainly by kinking and shortening of the tail (1, 3, 4). *Axin<sup>Ki</sup>* and *Axin<sup>Kb</sup>* also caused recessive embryonic lethality at E8–E10. A fourth allele, *Axin<sup>Tgl</sup>*, induced by a

random transgene insertion, had no dominant effects but caused recessive lethal embryonic defects similar to those observed in *Axin<sup>Ki/Ki</sup>* or *Axin<sup>Kb/Kb</sup>* embryos (5). Embryos homozygous for any of the recessive lethal alleles showed frequent neuroectodermal defects, including truncation or incomplete closure of the anterior neural folds, as well as cardiac defects. An intriguing feature of many homozygous embryos was a duplication of the embryonic axis, suggesting a role for *Axin* in embryonic axial development (3, 5, 6).

With the aid of the *Axin<sup>Tgl</sup>* insertional allele, the gene was cloned, and the wild type and mutant *Axin* alleles were characterized (2, 5, 7). The murine *Axin* gene is ubiquitously expressed in wild type embryos and adult tissues, encoding a major mRNA of ~4 kb<sup>1</sup> and a minor 3-kb mRNA. The ~4-kb mRNA is found in two isoforms that encode proteins of 956 (form 1) and 992 amino acids (form 2). Form 2 is identical to form 1 except for an insertion of 36 amino acids at position 856, due to alternative splicing. The Axin sequence revealed two regions of homology to other protein families as follows: an RGS domain (8, 9) at amino acids 213–338 and a “DIX domain” (10) at the extreme C terminus. The RGS domains of bona fide RGS (Regulation of G-protein Signaling) proteins bind to G $\alpha$ <sub>i</sub> proteins and serve as a GTPase-activating proteins (reviewed in Ref. 11). However, the Axin RGS domain differed from the consensus at most of the residues that make important contacts with the G $\alpha$ <sub>i</sub> switch regions (12), suggesting that it probably has a different function. The DIX domain is a region of similarity between the N terminus of Disheveled proteins (*Drosophila* Dsh and its vertebrate homologs) and the C terminus of Axin (10). Whereas truncation of a 165-amino acid N-terminal segment of Dsh, including this domain, abolished its activity in a *Drosophila* cell culture assay for Wingless signaling (13), the specific role of this domain remains obscure. Thus, the sequence of Axin provided few clues as to its function.

In both the *Axin<sup>Tgl</sup>* and *Axin<sup>Kb</sup>* alleles, synthesis of the full-length mRNAs is precluded by a transgene insertion in the former (2, 5) and a retroviral insertion in the latter (7). As both of these alleles caused axial duplication, it was suggested that *Axin* normally plays a negative regulatory role in the response to an axis-inducing signal in early mouse embryogenesis. This hypothesis was supported by the ability of Axin mRNA to block dorsal axis formation, *i.e.* to “ventralize,” when injected into early *Xenopus* embryos. Further analyses revealed that this ability is due to the inhibitory effect of Axin on a Wnt signaling pathway required for dorsal axis formation (2). This signaling pathway, which is closely related to the wingless signaling

\* This work was supported by a fellowship (to W. H.) from the National Kidney Foundation and by grants (to F. C.) from the National Institutes of Health. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EBI Data Bank with accession number(s) AF076192.

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<sup>1</sup> The abbreviations used are: kb, kilobase pair(s); APC, adenomatous polyposis coli; PP2A, protein phosphatase 2A; GST, glutathione S-transferase; RGS, regulation of G-protein signaling; CMV, cytomegalovirus; PAGE, polyacrylamide gel electrophoresis; HA, hemagglutinin.

pathway of *Drosophila*, includes GSK-3, a serine/threonine kinase also involved in glycogen metabolism, and  $\beta$ -catenin, a protein also involved in cell adhesion (reviewed in Ref. 14). When active, GSK-3 can phosphorylate  $\beta$ -catenin (15), leading to its degradation through the ubiquitin-dependent proteolysis system (16). In the presence of certain Wnts, GSK-3 is inhibited (through an unknown mechanism involving the cytoplasmic protein Dsh), allowing  $\beta$ -catenin to accumulate in the cytosol and to interact with transcription factors of the LEF/Tcf family (17, 18).  $\beta$ -Catenin and LEF/Tcf then translocate to the nucleus, where they bind to DNA and activate target genes, which, in the early *Xenopus* embryo, include the homeobox gene *Siamois* (19–21). Through co-injection experiments, Axin was found to inhibit this signaling pathway at a level downstream of Wnt, Dsh, and GSK-3 but upstream of  $\beta$ -catenin and *Siamois*. Thus, it was proposed that Axin, directly or indirectly, stimulated the phosphorylation of  $\beta$ -catenin by GSK-3 (2).

This prediction has been supported by several recent studies, which showed that Axin binds directly to GSK-3 and  $\beta$ -catenin, through distinct domains at amino acids 477–561 and 561–630, respectively (22–25). By simultaneously binding GSK-3 $\beta$  and  $\beta$ -catenin, Axin appears to promote the phosphorylation of  $\beta$ -catenin on serine/threonine residues. Axin also binds to APC, another protein implicated in the regulation of  $\beta$ -catenin (26–28), through its RGS domain (22). The role of APC binding in the function of Axin remains unclear; a truncated Axin lacking the entire N terminus, including the RGS domain, still promoted the turnover of  $\beta$ -catenin (22) in mammalian cells, whereas an internal deletion of only the RGS domain abolished the ventralizing ability of Axin in *Xenopus* embryos (2).

So far, the role of the C-terminal third of Axin (beyond the  $\beta$ -catenin binding region) is unclear. To identify proteins that interact with this region of Axin, we performed a yeast two-hybrid screen using the C-terminal 324 amino acids. We have thus identified a region of Axin that binds to the serine-threonine phosphatase PP2A. Our results suggest that PP2A may interact with the complex containing Axin, APC, GSK-3 and  $\beta$ -catenin, where it could serve to antagonize the effects of the kinase GSK-3. We also identified a C-terminal region of Axin that can bind to itself, suggesting that the protein may exist as a dimer or multimer.

#### EXPERIMENTAL PROCEDURES

**Recombinant Plasmids**—The bait plasmid pGBT9-Axin-(632–956) was constructed by inserting the *Pst*I fragment of Axin cDNA (form 1) into the *Pst*I site of pGBT9 vector (CLONTECH) to generate a Gal4DB-Axin fusion protein in yeast. This plasmid was sequenced to confirm that the coding sequences for Gal4DB and Axin were in frame. A series of pGBT9-Axin plasmids containing different regions of the Axin cDNA (Fig. 5) were created using convenient restriction enzyme sites. A method to create unidirectional nested deletions of double-stranded DNA clones using exonuclease III and mung bean nuclease was also performed to generate pGBT9-Axin-(632–910) and pGBT9-Axin-(632–836) (Exo-Size Deletion Kit, New England Biolabs). To express N-terminal FLAG-tagged Axin proteins in 293T cell, DNA fragments containing different regions of FLAG-Axin cDNA were cloned into a mammalian expression vector containing a CMV promoter (pcDNA3, Invitrogen). The pCMV7-p36 plasmid contains a full-length PP2A<sub>c</sub> cDNA that is tagged at the N terminus with T7 and inserted into the pcDNA3 vector. The pCMVHA-PR65 plasmid contains a full-length regulatory A subunit of PP2A tagged with HA at N terminus (29). Three fragments of Axin cDNA were cloned downstream of the glutathione S-transferase (GST) gene to generate pGST-Axin-(421–810), pGST-Axin-(632–810), and pGST-Axin-(632–956) plasmids and to produce recombinant proteins. The PP2A<sub>c</sub> cDNA was inserted into a vector containing a translation initiation sequence and a FLAG-tag sequence to create the pBFT4-PP2A<sub>c</sub> plasmid for *in vitro* transcription and translation. pGADNOT-PP2A<sub>c</sub> contains a full-length mouse PP2A<sub>c</sub> cDNA cloned into a pGADNOT prey vector (30). The pGAD424-Axin-(194–956) plasmid contains an Axin cDNA fragment encoding amino acids 194–956 inserted into the pGAD424 prey vector (CLONTECH). The

pGAD-PR65 plasmid contains a cDNA fragment encoding the full-length regulatory A subunit of PP2A cloned into the pGAD prey vector (31).

**Yeast Two-hybrid System**—The pGBT9-Axin-(632–956) plasmid was co-transformed with an expression library consisting of cDNAs from a murine macrophage cell line WEHI-3, which were cloned into the pGADNOT prey vector (30), into the yeast Y190 strain. Yeast transformants were grown on synthetic medium lacking leucine, tryptophan, and histidine. The expression of *his* and *lacZ* reporter genes were used to assay for clones encoding proteins that associate with Axin. The positive colonies were then grown on synthetic medium lacking only leucine to lose the pGBT9-Axin-(632–956) plasmid and to test for absence of  $\beta$ -galactosidase activity in the absence of the bait vector. To recover the pGADNOT plasmids, extracts of plasmid DNA from yeast were introduced into *Escherichia coli* JM83 by electroporation. Each of the pGADNOT plasmids was then co-transformed with pGBT-Axin-(632–956) plasmid into yeast Y190. Transformants were grown on synthetic medium lacking leucine and tryptophan and tested the ability to bind Axin in the  $\beta$ -galactosidase filter assay. Inserts of cDNA clones showing interaction with pGBT9-Axin-(632–956) were sequenced.

**In Vitro Binding Assay with GST-Axin**—pGST-Axin-(421–810), pGST-Axin-(632–810), and pGST-Axin-(632–956) plasmids were used to express and purify recombinant GST fusion proteins as described previously (32). PP2A<sub>c</sub> RNA was transcribed *in vitro* from pBFT4-PP2A<sub>c</sub> using T7 RNA polymerase. PP2A<sub>c</sub> protein was translated with reticulocyte lysate and labeled with [<sup>35</sup>S]methionine *in vitro* (Promega). *In vitro* binding of GST-Axin and PP2A<sub>c</sub> was essentially as described (32). The labeled PP2A<sub>c</sub> proteins were incubated with GST or GST-Axin proteins in the association buffer, and protein complexes were precipitated with glutathione-Sepharose and analyzed by SDS-PAGE and autoradiography.

**Transfection**—293T cells were transfected by a calcium phosphate-mediated transfection method (33) at 24 h after plating ( $2.0 \times 10^6$  cells per 100-mm dish). Ten mg of each plasmid DNA was used in each reaction, and sonicated salmon sperm DNA was used as a supplement to maintain the same DNA concentration in each transfection. Approximately 48 h after transfection, cells were lysed for protein expression and binding analyses.

**Immunoblot Analysis**—Protein extracts or immunoprecipitated complexes were subject to immunoblotting as described (34). Anti-FLAG (Kodak), anti-T7 conjugated with alkaline phosphatase (Novagen), or anti-Myc (Calbiochem) monoclonal antibodies were used to analyze the presence of tagged proteins in transfected cells. Except for anti-T7, bound antibodies were then detected with a goat anti-mouse IgG alkaline phosphatase-conjugated secondary antibody (American Qualex) and visualized by the chromogenic substrate reaction.

**Co-immunoprecipitation Analysis**—293T cells were lysed in a buffer containing 50 mM Tris-HCl (pH 8.0), 200 mM NaCl, 20 mM NaF, 20 mM  $\beta$ -glycerophosphate, 100 mM sodium vanadate, 0.1 mM phenylmethylsulfonyl fluoride, 1 mM dithiothreitol, 2 mg/ml leupeptin, 2 mg/ml antipain, 0.1% Triton X-100, and 0.5% Nonidet P-40. Protein complexes were immunoprecipitated with monoclonal anti-FLAG or polyclonal anti-PP2A<sub>c</sub> antibody (Promega), followed by immunoblotting analysis.

#### RESULTS

**Identification of Axin-binding Proteins**—A cDNA sequence encoding a C-terminal segment of Axin (amino acids 632–956, form 1) was cloned into the pGBT9 bait vector to produce a Gal4-DNA-binding domain-Axin fusion protein for yeast two-hybrid screening (35). Because Axin is expressed in most if not all cell types, a murine macrophage cDNA library cloned in the pGADNOT prey vector was screened to identify proteins that can interact with Axin. After co-transformation of pGBT9-Axin-(632–956) with the expression library, 12 colonies out of approximately 50,000 potential transformants survived on synthetic medium lacking histidine (Table I, 1<sup>o</sup> screen). The growth of these colonies implied that they contained clones encoding proteins that bound to Axin-(632–956) and also suggested the expression of the *his* reporter gene under the control of a Gal4-responsive promoter in these transformants. The expression of a second Gal4-responsive reporter gene,  $\beta$ -galactosidase, was found in 9/12 of these colonies (Table I, 2<sup>o</sup> screen). All nine of these clones tested negative for  $\beta$ -galactosidase in the absence of the bait vector pGBT9-Axin-(632–956) (Table I, 3<sup>o</sup> screen).

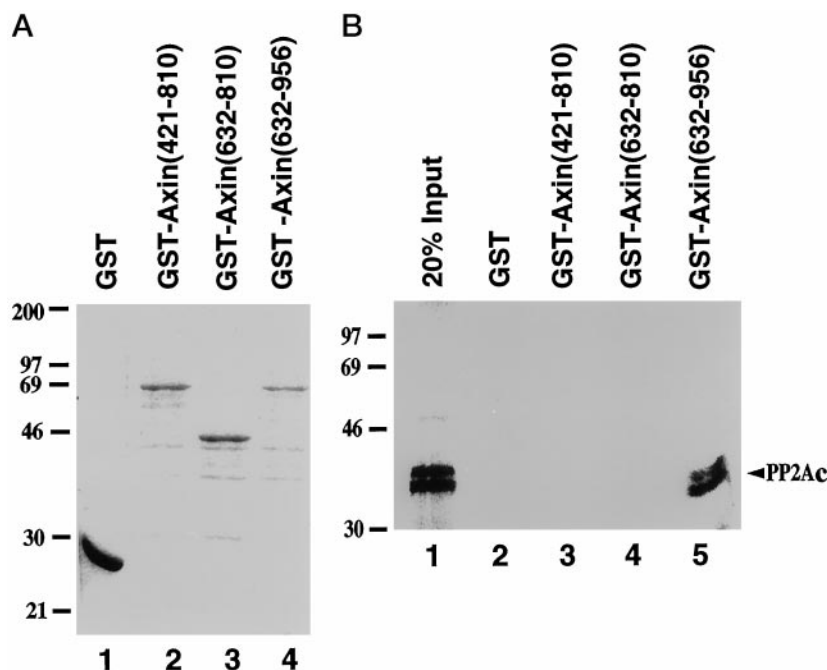
The pGADNOT prey plasmids were recovered from these nine transformants. Only three of them exhibited the ability to

TABLE I  
Summary of identification of Axin-binding proteins by yeast two-hybrid screening

The primary selection was the growth of yeast transformants on histidine<sup>-</sup> medium (1°), followed by the expression of a second reporter gene (2°),  $\beta$ -galactosidase ( $\beta$ -Gal). The 3° screen was done by losing the bait pGBT9-Axin-(632–956) plasmid in those transformants. Finally, pGADNOT prey plasmids were recovered and cotransformed with the bait vector containing either Gal4DB or Gal4DB-Axin to reconstitute the expression of the  $\beta$ -galactosidase reporter gene (4°). +, positive; -, negative; NA, not applicable.

Screening/clone no.	1	2	3	4	5	6	7	8	9	10	11	12
1°-his+	+	+	+	+	+	+	+	+	+	+	+	+
2°- $\beta$ -Gal+	+	+	+	+	+	+	+	+	-	-	+	-
3°-No bait plasmid, $\beta$ -Gal+	-	-	-	-	-	-	-	-	NA	NA	-	NA
4°-Reconstitution, +Gal4DB	-	-	-	-	-	-	-	-	NA	NA	-	NA
+Gal4DB-Axin	-	-	+	-	-	-	+	-	NA	NA	+	NA

FIG. 1. Binding of Axin to the catalytic subunit of PP2A (PP2A<sub>c</sub>) *in vitro*. A, Coomassie Blue staining of purified recombinant glutathione *S*-transferase (GST, lane 1) and three GST-Axin proteins (lanes 2–4). Numbers in parentheses indicate the corresponding amino acids of Axin protein (form 1). The migration of protein molecular weight markers is shown on the left. B, association of Axin with PP2A<sub>c</sub> *in vitro*. PP2A<sub>c</sub> (p36) was translated *in vitro* with reticulocyte lysate in the presence of [<sup>35</sup>S]methionine and incubated with the recombinant proteins shown in A. The protein complexes were precipitated with glutathione-Sepharose and analyzed by SDS-PAGE and autoradiography. The 1st lane contains <sup>35</sup>S-labeled PP2A<sub>c</sub> in a quantity equivalent to 20% of the input for each binding reaction, analyzed directly by SDS-PAGE and autoradiography.



bind to Axin upon co-transformation with pGBT9-Axin-(632–956) in the yeast two-hybrid system (Table I, 4° screen). Two of the three plasmids contained a 1.9-kb DNA insert and the other a 1.3-kb insert. DNA sequence analysis of the two 1.9-kb clones showed that they were identical and encoded a mouse protein displaying 99% amino acid identity to the  $\alpha$  isoform of the catalytic subunit of rat and human serine/threonine protein phosphatase 2A (PP2A<sub>c</sub>).<sup>2</sup> The 1.3-kb clone encoded a C-terminal segment of Axin protein (amino acids 831–956 of form 1). These data suggest that Axin binds to PP2A<sub>c</sub> and also associates with itself through the C-terminal region.

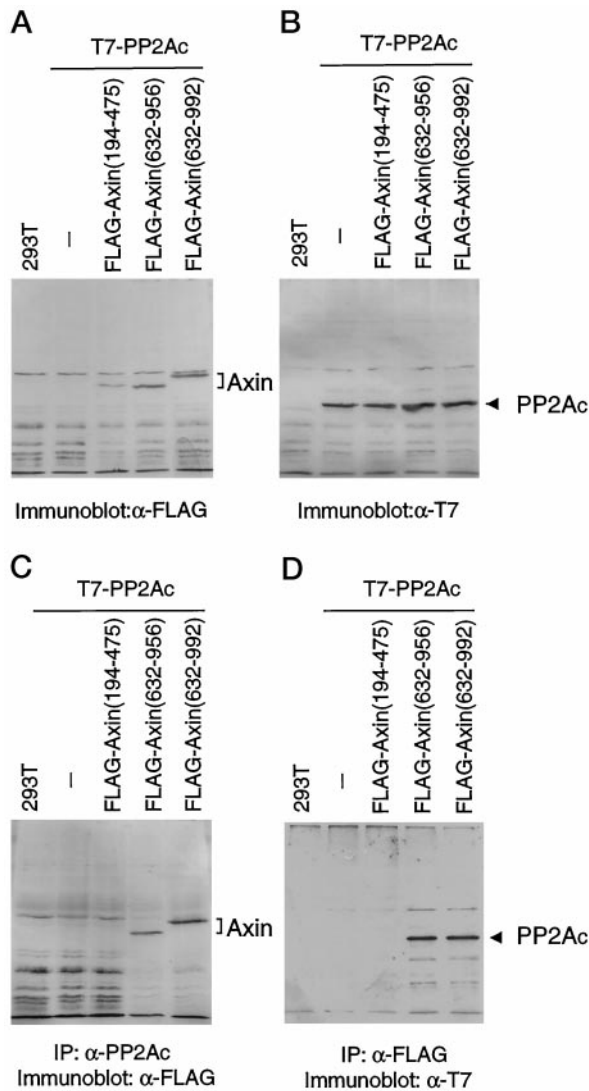
**Axin Interacts with PP2A<sub>c</sub> *In Vitro***—PP2A is a heterotrimeric enzyme consisting of a catalytic subunit (C) associated with a 65-kDa regulatory subunit (A) and a third variable subunit (B) (36–38). In mammals, the closely related  $\alpha$  and  $\beta$  isoforms of PP2A<sub>c</sub> are encoded by separate genes but are indistinguishable in function. Axin contains several predicted sites for Ser/Thr phosphorylation (2), and Ser/Thr phosphorylation of  $\beta$ -catenin is thought to play a critical role in Wnt signaling (14, 15). This suggested that the Axin-PP2A<sub>c</sub> interaction might be biologically significant. Therefore, the ability of PP2A<sub>c</sub> to interact physically with Axin was independently examined by an *in vitro* binding assay (Fig. 1). Three different recombinant GST-Axin fusion proteins and control GST protein were bacterially expressed and purified (Fig. 1A) and were incubated with

*in vitro* synthesized, <sup>35</sup>S-labeled PP2A<sub>c</sub>. Analysis of protein complexes precipitated with glutathione-Sepharose indicated that PP2A<sub>c</sub> bound to a C-terminal region of Axin, amino acids 632–956 (Fig. 1B, lane 5). However, two fusion proteins lacking the last 146 amino acids, GST-Axin-(421–810) and GST-Axin-(632–810), failed to bind PP2A<sub>c</sub> (Fig. 1B, lanes 3 and 4).

**Axin Co-immunoprecipitates with PP2A<sub>c</sub>**—To examine the association of Axin and PP2A<sub>c</sub> *in vivo*, T7 epitope-tagged PP2A<sub>c</sub> and three different FLAG-tagged Axin proteins were transiently expressed in 293T cells (Fig. 2, A and B). PP2A<sub>c</sub> was co-immunoprecipitated with FLAG-Axin-(632–956), which contains a C-terminal polypeptide identical to the one used as bait in the yeast two-hybrid screen, but not with FLAG-Axin-(194–475), which includes the RGS domain (Fig. 2C). The FLAG-Axin-(632–992), derived from form 2 of Axin, also co-precipitated with PP2A<sub>c</sub>, suggesting that both isoforms of Axin interact with PP2A<sub>c</sub> regardless of the 36 amino acids insertion (Fig. 2C). Similar results were also obtained when cell lysates were first immunoprecipitated with anti-FLAG antibody followed by immunoblotting with anti-T7 antibody (Fig. 2D). These data agree with the conclusions from yeast two-hybrid and *in vitro* biochemical analyses, confirming the ability of Axin and PP2A<sub>c</sub> to interact *in vivo*.

**Axin Directly Associates with the PP2A<sub>c</sub> Catalytic Subunit**—The interaction of Axin and PP2A<sub>c</sub> in various experimental assays raised the question whether Axin binds directly to the PP2A<sub>c</sub> subunit or whether it might associate indirectly with PP2A<sub>c</sub> by binding to the regulatory A subunit, which itself

<sup>2</sup> The nucleotide sequence for the mouse PP2A<sub>c</sub> gene has been deposited under accession number AF076192.



**FIG. 2. Co-immunoprecipitation of Axin with PP2A<sub>c</sub>.** PP2A<sub>c</sub> was cloned into a CMV expression vector encoding a T7 epitope tag (T7-PP2A<sub>c</sub>), and three Axin cDNA fragments were cloned into a similar vector encoding a FLAG epitope tag. FLAG-Axin-(632–956) contained sequences from Axin form 1; FLAG-Axin-(632–992) contained sequences from Axin form 2, with a 36-amino acid insertion at position 856. 293T cells were either lysed without transfection (293T lanes) or transfected with T7-PP2A<sub>c</sub> alone (– lanes) or with T7-PP2A<sub>c</sub> plus one of the three FLAG-Axin plasmids. *A*, cell lysates were immunostained with FLAG antibody, showing the expression of three FLAG-Axin proteins. *B*, cell lysates were immunoblotted with T7 antibody, showing the expression of T7-PP2A<sub>c</sub>. Protein complexes were either immunoprecipitated (*IP*) with PP2A<sub>c</sub> antibody, separated by SDS-PAGE, and immunoblotted with FLAG antibody (*C*), or immunoprecipitated with FLAG antibody, separated by SDS-PAGE, and immunoblotted with T7 antibody (*D*). The FLAG-Axin-(632–956) and FLAG-Axin-(632–992), but not FLAG-Axin-(194–475), co-precipitated with T7-PP2A<sub>c</sub> from 293T cells. The presence of FLAG-Axin and T7-PP2A<sub>c</sub> proteins was visualized by an alkaline phosphatase-mediated chromogenic substrate reaction. The brackets indicate the Axin proteins and arrowheads the PP2A<sub>c</sub> protein.

binds tightly to the catalytic subunit. A co-immunoprecipitated assay was first performed to test whether the regulatory A subunit was present in the Axin-PP2A<sub>c</sub> complex. The T7-tagged PP2A<sub>c</sub> and HA-tagged PR65 (regulatory A subunit of PP2A) were transiently expressed together with four different Myc- or Flag-tagged Axin proteins in 293T cells (Fig. 3, *A* and *B*). PR65 only co-precipitated with Axin proteins that contain the PP2A-binding domain, indicating that PR65 can indeed associate with Axin *in vivo* (Fig. 3*C*). Next, the yeast two-hybrid assay was used to analyze further the ability of Axin to bind to PR65.

Unlike the catalytic subunit, PR65 failed to show any interaction with Axin in this assay (Table II). We therefore conclude that Axin can bind directly to the PP2A<sub>c</sub> catalytic subunit and only indirectly to PR65.

**Self-interaction of Axin Proteins**—The cloning of a fragment of Axin by the yeast two-hybrid screen with pGBT9-Axin-(632–956) (Table I) raised the possibility that Axin may form dimers or high order complexes with itself. The self-association of Axin was tested using the co-immunoprecipitated assay (Fig. 4). A Myc epitope-tagged Axin-(811–956), which could be distinguished from FLAG-tagged Axin proteins, was transiently expressed in 293T cells (Fig. 4*A*). The FLAG-Axin proteins were expressed simultaneously (Fig. 4*B*) and tested for co-immunoprecipitation (Fig. 4*C*). The results confirmed that Axin can interact with itself through a region located near the extreme C terminus. The two isoforms of Axin were both capable of association with each other and with themselves.

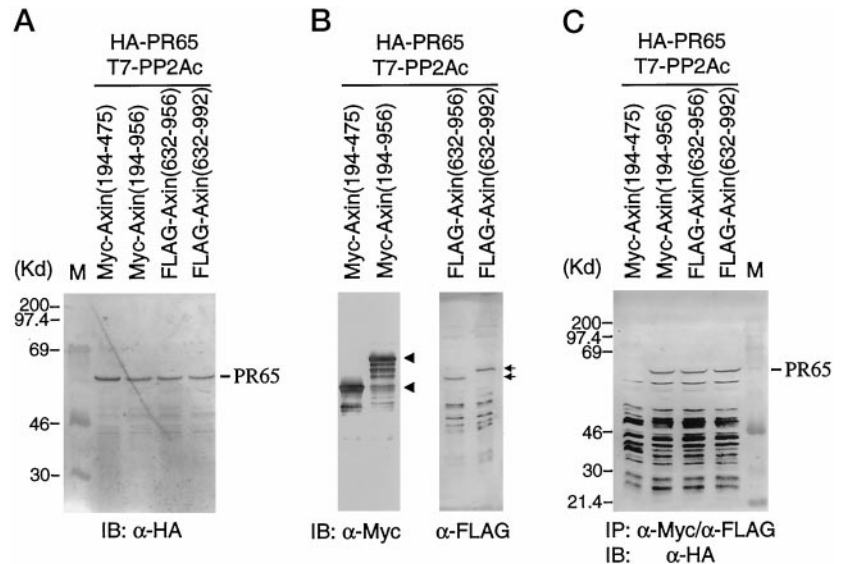
**Localization of the PP2A<sub>c</sub>-binding and Self-binding Domains of Axin**—To delineate the regions of Axin capable of mediating self-association and association with PP2A<sub>c</sub>, a series of deletion mutants of Axin was generated for yeast two-hybrid analysis. This experimental system was used because of its high sensitivity to detect protein-protein interactions (Fig. 5). Whereas Axin-(632–836) interacted strongly with PP2A<sub>c</sub>, deletion of amino acids 811–836 greatly reduced this interaction, suggesting the C-terminal boundary of the PP2A<sub>c</sub>-binding domain is within this 26-amino acid region. Similarly, the N-terminal boundary of the PP2A<sub>c</sub>-binding domain was found to reside between amino acids 632 and 744. The weak interaction between Axin-(632–810) and PP2A<sub>c</sub> detected in the yeast two-hybrid analysis was not observed in the *in vitro* biochemical assay (Fig. 1*B*, lanes 3 and 4), which may reflect a different sensitivity of the two assays. Whereas other regions outside this domain are not required for PP2A<sub>c</sub> binding, an N-terminal region, including the RGS domain, seemed to reduce the ability of Axin-(194–956) to interact with PP2A<sub>c</sub>.

A similar serial deletion mutant analysis revealed that the self-binding domain of Axin is distinct from the PP2A<sub>c</sub>-binding domain and is located at the extreme C terminus (Fig. 5). Both isoforms of Axin are equally potent for self-association (compare Axin-(632–956) and Axin-(632–992)), suggesting that the self-binding domain is C-terminal to the insertion in form 2, *i.e.* within the last 100 amino acids. Moreover, the DIX domain (amino acids 899–949 of form 1, and 935–985 of form 2) is required for self-association, because deletion of amino acids 910–956 completely abolished this activity. In contrast to PP2A<sub>c</sub>-binding, the self-binding of the Axin C terminus was not affected by the presence of N-terminal sequences (Axin-(194–956)).

## DISCUSSION

Axin has been shown to serve as a component of a Wnt signal transduction pathway that involves the phosphorylation of  $\beta$ -catenin by the serine/threonine kinase GSK-3. The involvement of Axin in this pathway was originally demonstrated based on its ability to inhibit dorsal axis formation in *Xenopus* embryos (2). mRNAs encoding certain members of the Wnt family, or several other proteins that function in the signaling pathway downstream from these Wnts, can induce an ectopic dorsal axis when injected ventrally in the early embryo (14). Axin appeared to function downstream of GSK-3 and upstream of  $\beta$ -catenin, as its effect on axis formation could be overcome by co-injection of  $\beta$ -catenin or the transcription factor Siamois but not by co-injection of Wnt8, Dsh, or a dominant-negative mutant form of GSK-3 (2). Recently, it has been demonstrated that Axin binds directly to both GSK-3 and  $\beta$ -catenin, suggesting a biochemical basis for its effects on signaling through this pathway (22–25). Axin also binds, through its RGS domain, to

**FIG. 3. Co-immunoprecipitation of Axin with the regulatory A subunit of PP2A.** HA-tagged A subunit (PR65) and T7-tagged PP2A<sub>c</sub> were transiently co-expressed with four different fragments of Axin protein, which were either Myc- or Flag-tagged, in 293T cells. Lysates from transfected cells were immunoblotted (IB) with either  $\alpha$ -HA antibody, to detect the expression of PR65 (A), or  $\alpha$ -Myc and  $\alpha$ -Flag antibodies (arrowheads and arrows, respectively) to detect the expression of Axin fragments (B). Protein complexes were immunoprecipitated (IP) with either  $\alpha$ -Myc or  $\alpha$ -Flag antibody, followed by immunoblotting with  $\alpha$ -HA antibody (C). Axin-(194–956), -(632–956), and -(632–992) fragments but not Axin-(194–475) co-precipitated with PR65 as indicated. M represents the protein molecular weight markers.



**TABLE II**  
The regulatory A subunit of PP2A, PR65, does not interact with Axin in the yeast two-hybrid system.

In a control experiment, PR65 was able to interact with PP2A<sub>c</sub>, its normal partner, in this assay. +++, dark blue developed after 5 h of  $\beta$ -galactosidase ( $\beta$ -Gal) assay; -, only white color after 12 h of  $\beta$ -galactosidase assay.

Bait	Prey	$\beta$ -Gal filter assay
Axin-(632–956)	PP2A <sub>c</sub>	+++
PP2A <sub>c</sub>	PR65	+++
Axin-(194–956)	PR65	-
Axin-(632–956)	PR65	-
Axin-(632–992)	PR65	-

APC, a protein previously shown to bind to  $\beta$ -catenin and promote its degradation (22). In this study, we have used the yeast two-hybrid system, together with *in vitro* binding and *in vivo* co-immunoprecipitation assays, to identify two additional protein-binding domains of Axin. A region of Axin C-terminal to the APC, GSK-3 and  $\beta$ -catenin-binding domains, between amino acids 632 and 836, can bind to the catalytic subunit of the Ser/Thr protein phosphatase PP2A, whereas a sequence at the extreme C terminus of Axin (amino acids 856–956), including the conserved DIX domain, can bind to the same region of other Axin molecules (Fig. 6). Processes that are regulated by protein phosphorylation require a protein phosphatase to modulate the effects of a protein kinase (39). Thus, the ability of Axin to bind to PP2A suggests that this phosphatase can interact with the Axin-APC-GSK-3- $\beta$ -catenin complex, where it might modulate the effect of GSK-3 on  $\beta$ -catenin or other substrates. Potentially, Axin could play a role in other signaling pathways involving PP2A. The ability of Axin to bind to itself suggests that it may function as a dimer or higher order multimer.

PP2A, one of the four major classes of Ser/Thr protein phosphatases, is a heterotrimeric enzyme whose core dimer consists of a 36-kDa catalytic subunit C and a 65-kDa regulatory subunit A. This core dimer can associate with one of several regulatory B subunits ranging in size from 54 to 130 kDa. PP2A is widely expressed and has broad substrate specificity *in vitro* (36–38). Genetic studies in yeast and *Drosophila*, as well as experiments in *Xenopus* oocytes and mammalian cells, have implicated PP2A in a wide range of biological processes, including cell division, transformation, cell cycle regulation, cell fate determination, and gene expression (40, 41). However, only a few of the specific *in vivo* substrates of PP2A have been iden-

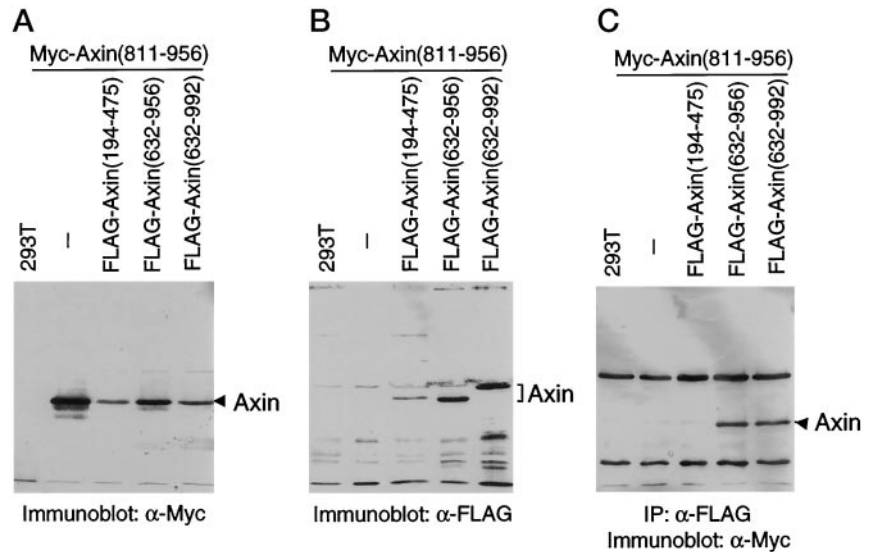
tified. These are believed to include the neuronal microtubule-associated protein tau (42), several components of the mitogen-activated protein kinase cascade (41, 43, 44), the atypical protein kinase C  $\zeta$  (45) and Ca<sup>2+</sup>-calmodulin-dependent protein kinase IV (46). The function of the regulatory B subunits is not fully understood, but they are believed to influence substrate specificity and possibly subcellular localization of PP2A (47, 48). In addition, several other proteins have been shown to bind to the PP2A catalytic subunit and regulate its activity or localization, including viral tumor antigens (49), the translation termination factor eRF1 (50), the Ser/Thr kinase casein kinase 2 $\alpha$  (51), and the homeobox protein Hox11 (52). The ability of Axin to bind to PP2A suggests that Axin might be yet another regulatory protein for PP2A, which could influence any of the various roles of PP2A in the cell. However, the established role of Axin in the Wnt pathway suggests a novel role for PP2A in this pathway.

The phosphorylation of  $\beta$ -catenin (or Armadillo) by GSK-3 (or shaggy/zeste-white) appears to be critical step in the conserved signaling pathway downstream from *Drosophila* wingless and certain members of the vertebrate Wnt family (14). Axin appears to promote this phosphorylation event by simultaneously binding to GSK-3 and  $\beta$ -catenin (22–25). Wnts, which are believed to bind to receptors of the Frizzled family, inhibit GSK-3 activity through an unknown mechanism involving the cytoplasmic protein Dsh (reviewed in Ref. 10). This explains why these Wnts, Dsh, or dominant-negative GSK-3 can induce a dorsal axis when their mRNAs are injected into *Xenopus* embryos (reviewed in Ref. 14). However, the failure to identify a Wnt that is expressed in the right time and place to induce axis formation in the *Xenopus* embryo has led to the suggestion that some other mechanism may trigger signal transduction through this pathway in the normal amphibian embryo (14, 53, 54).

The ability of Axin to bind to the Ser/Thr phosphatase PP2A raises the possibility that PP2A might play a role in signal transduction through  $\beta$ -catenin, by opposing the effect of the kinase GSK-3. Binding to Axin would recruit PP2A to the Axin-APC-GSK-3- $\beta$ -catenin complex, where it could dephosphorylate Ser/Thr residues on  $\beta$ -catenin (as well as other phosphoproteins in the complex). Recent data show that the PP2A-binding region of Axin is not required for its ventralizing effect in the frog embryo (23).<sup>3</sup> This indicates that the ability of Axin

<sup>3</sup> F. Fagotto, E.-H. Jho, L. Zeng, and F. Costantini, unpublished data.

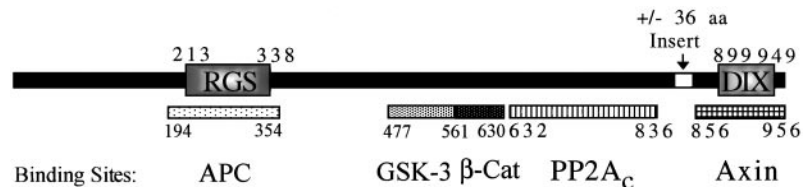
**FIG. 4. Axin self-association detected by co-immunoprecipitation.** A vector encoding Myc-tagged Axin-(811–956) was transfected into 293T cells alone (– lanes) or together with one of three FLAG-tagged Axin vectors. Cell lysates from non-transfected cells (293T lanes) or transfected cells were subject to immunostaining and immunoprecipitation-immunoblot analyses. Cell lysates were immunostained with  $\alpha$ -Myc antibody, showing the expression of Myc-tagged Axin-(811–956) (A), or immunostained with  $\alpha$ -FLAG antibody, showing the expression of FLAG-tagged Axin proteins (B). Protein complexes were immunoprecipitated (IP) with  $\alpha$ -FLAG, followed by immunoblotting with  $\alpha$ -Myc (C). FLAG-Axin-(632–956) and -(632–992) but not -(194–475) were capable of self-association with Myc-Axin-(811–956). Signal above and below the arrowhead is due to alkaline phosphatase-conjugated secondary antibody reacting with primary antibody used in immunoprecipitation.



**fragment of Axin protein**

fragment of Axin protein		yeast 2-Hyb. interaction with		
		PP2A <sub>C</sub>		Axin
		filter / liquid assay		filter assay
194	956	+	18%	+++
476	956	+++	100%	+++
531	956	+++	90%	+++
632	956	+++	98%	+++
632	992	+++	99%	+++
744	956	–	2%	+++
831	956	–	ND	+++
632	910	+++	ND	–
632	836	+++	ND	–
632	810	+	12%	–
632	744	–	2%	–
476	632	–	2%	–
531	632	–	2%	–
194	531	–	4%	–

**FIG. 6. Schematic representation of Axin protein, showing the RGS and DIX homology domains, and binding regions for APC, GSK-3,  $\beta$ -catenin ( $\beta$ -Cat), PP2A<sub>C</sub>, and Axin.** See text for details and references. aa, amino acid.



**FIG. 5. Determination of PP2A<sub>C</sub> binding and Axin self-binding domains in the yeast two-hybrid (yeast 2-Hyb.) system.** The schematic representation indicates the segments of Axin protein tested for interaction with PP2A<sub>C</sub> or with Axin-(194–965). The  $\beta$ -galactosidase filter assay was performed on Leu<sup>–</sup>, Trp<sup>–</sup> plates, and intensity of color was scored after 5 h at 30 °C. +++, dark blue color developed in <2 h; +, light blue color developed in >5 h; –, no blue color after >5 h. For the  $\beta$ -galactosidase liquid assay, values are stated as a percentage of the highest value observed, for Axin-(476–956). Yeast co-transformed with void bait (pGBT9) and prey (pGAD424) vectors were used to determine the background level for the liquid assay. ND, not determined.

to negatively regulate this signaling pathway when overexpressed does not depend on its ability to bind to PP2A. On the contrary, PP2A might modulate the tendency of Axin to promote the phosphorylation of  $\beta$ -catenin by GSK-3. According to this model, the signal initiating embryonic axis formation might lead to the recruitment of PP2A to the Axin-APC-GSK-3- $\beta$ -catenin complex, leading to the dephosphorylation and stabilization of  $\beta$ -catenin. This model predicts that a truncated Axin protein lacking the PP2A-binding domain would be hyperactive, since it could stimulate the GSK-3/ $\beta$ -catenin interaction without allowing modulation by PP2A. No clear-cut difference in the ventralizing activity of full-length Axin versus such a truncated Axin has been observed.<sup>2</sup> However, this assay,

in which large quantities of Axin are expressed in the embryo, may be insensitive to the ability of Axin to bind PP2A (for example, the amount of Axin expressed may greatly exceed the amount of available PP2A in the embryo).

Evidence that is consistent with this model comes from studies of two spontaneous mutant alleles of Axin, Axin<sup>Fu</sup> and Axin<sup>Kb</sup>. These two alleles, which have similar dominant phenotypic effects, both produce abnormally spliced transcripts as a result of retroviral insertions into nearby regions of the gene. These abnormal transcripts are predicted to encode proteins with C-terminal truncations, terminating at residue 720 (Axin<sup>Fu</sup>) or 766 (Axin<sup>Kb</sup>). Whereas these truncated proteins would retain the binding domains for APC, GSK-3, and  $\beta$ -catenin, their ability to

bind PP2A would be either abolished or greatly reduced (Fig. 4). It has been previously concluded that the dominant effects of these alleles are due to gain-of-function rather than loss-of-function effects, since a deletion that removes the entire *Axin* gene (by definition, a loss-of-function mutation) has no dominant effect (55). This is consistent with the observation that *Axin* constructs encoding proteins with C-terminal truncations at amino acid 724 (23) or 810<sup>3</sup> are active in the frog embryo ventralization assay and therefore do not appear to be loss-of-function mutants. However, the gain-of-function effects of *Axin*<sup>Fu</sup> and *Axin*<sup>Kb</sup> could be explained if the PP2A-binding region were a regulatory domain, whose removal resulted in a hyperactive form of Axin. Interestingly, *vestigial tail* (*vt*), a hypomorphic allele of mouse *Wnt-3a*, causes a recessive phenotype characterized by kinking and shortening of the tail (56), very similar to the dominant phenotype of *Axin*<sup>Fu</sup> or *Axin*<sup>Kb</sup>. *Wnt-3a* is normally expressed in the tail bud mesoderm and is required for caudal somitogenesis (57). Since *Wnt-3a* is one of the Wnt family members that signal through the GSK-3/ $\beta$ -catenin pathway (58, 59), Axin is likely to function as a negative regulator of *Wnt-3a* signal transduction during tail bud development. Therefore, a hypermorphic allele of *Axin* would be expected to phenocopy a hypomorphic allele of *Wnt-3a*.

The self-binding domain of Axin has been localized to a region of ~100 amino acids at the extreme C terminus, which is distinct from the PP2A-binding region. The self-binding region includes the DIX domain, a 51-amino acid region of similarity between Axin and Dsh proteins. This overlap raises the possibility that Dsh, or its vertebrate homologs, might also associate with Axin through the DIX domain. The ability of Axin to bind to itself through the C terminus also suggests that it might normally form dimers or higher order multimers in the cell. Axin is made in two major isoforms, which differ by an insertion of 36 amino acids at position 856; in addition, a smaller protein identical at the C terminus, but missing the N-terminal region, may be encoded by a minor 3-kb mRNA (2). Therefore, hetero- as well as homodimers of Axin may be formed. A different protein with extensive sequence similarity to Axin and apparently similar function has recently been identified (60, 61). The last 100 amino acids of this protein are 72% similar to those of Axin, suggesting that it might be able to dimerize with Axin.

What might be the importance of self-binding for the function of Axin? Perhaps dimerization of Axin enhances interactions between the other proteins that bind to Axin, e.g. phosphorylation by GSK-3 of  $\beta$ -catenin or other proteins, or de-phosphorylation by PP2A. A C-terminal truncated form of Axin lacking this region is active in the frog embryo ventralization assay (23),<sup>3</sup> implying that self-binding is not absolutely required for the ability of Axin to inhibit the Wnt signal transduction pathway. However, as discussed above, this assay may be insensitive to quantitative changes in the activity of Axin, and other assays may be required to observe the function of this domain.

**Acknowledgments**—We thank Kimona Alin and Stephen Goff for the expression library and Qin Ye and Howard Worman for advice on yeast two-hybrid screening.

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