Domains of Axin Involved in Protein–Protein Interactions, Wnt Pathway Inhibition, and Intracellular Localization

François Fagotto,* Eek-hoon Jho,‡ Li Zeng,† Thomas Kurth,* Thomas Joos,* Christine Kaufmann,* and Frank Costantini‡

*Division of Cell Biology, Max-Planck Institute for Developmental Biology, 72076 Tübingen, Germany; and ‡Department of Genetics and Development, College of Physicians and Surgeons, Columbia University, New York 10032

Abstract. Axin was identified as a regulator of embryonic axis induction in vertebrates that inhibits the Wnt signal transduction pathway. Epistasis experiments in frog embryos indicated that Axin functioned downstream of glycogen synthase kinase 3β (GSK3β) and upstream of β-catenin, and subsequent studies showed that Axin is part of a complex including these two proteins and adenomatous polyposis coli (APC). Here, we examine the role of different Axin domains in the effects on axis formation and β-catenin levels. We find that the regulators of G-protein signaling domain (major APC-binding site) and GSK3β-binding site are required, whereas the COOH-terminal sequences, including a protein phosphatase 2A binding site and the DIX domain, are not essential. Some forms of Axin lacking the β-catenin binding site can still interact indirectly with β-catenin and regulate β-catenin levels and axis formation. Thus in normal embryonic cells, interaction with APC and GSK3β is critical for the ability of Axin to regulate signaling via β-catenin. Myc-tagged Axin is localized in a characteristic pattern of intracellular spots as well as at the plasma membrane. NH2-terminal sequences were required for targeting to either of these sites, whereas COOH-terminal sequences increased localization at the spots. Coexpression of hemagglutinin-tagged Dsh revealed strong colocalization with Axin, suggesting that Dsh can interact with the Axin/APC/GSK3β complex, and may thus modulate its activity.

Key words: β-catenin • glycogen synthase kinase 3β (GSK3β) • adenomatous polyposis coli (APC) • Dsh • dorsal axis formation

Axin is the product of the murine genetic locus originally called Fused, in which mutations cause a variety of developmental defects (Gluessohn-Schoenheimer, 1949; Theiler and Gluecksohn-Waelsch, 1956; Jacobs-Cohen et al., 1984). The Axin gene was cloned with the aid of an insertional mutation and found to potentially encode a protein of up to 992 amino acids (aa)1 (Perry et al., 1995; Zeng et al., 1997). Axin contains two conserved domains, a regulators of G-protein signaling domain (RGS; Dohlman and Thorner, 1997) near its NH2 terminus and a COOH-terminal DIX domain (also found in Dsh; Cadigan and Nusse, 1997) that suggested a role in signal transduction. More specific insight into the function of Axin came from studies of its effects on vertebrate embryogenesis. The occurrence of axial duplications in loss-of-function Axin mutants in the mouse suggested that the gene might play a negative regulatory role in an early step in axis formation. This hypothesis was tested and confirmed by the ability of overexpressed Axin to block dorsal axis formation in Xenopus embryos. Further studies showed that the effect of Axin is due to its specific ability to inhibit signal transduction components of the Wnt pathway and suggested
that Axin functioned downstream of glycogen synthase kinase 3β (GSK 3β) and upstream of β-catenin (Zeng et al., 1998).

β-Catenin is thought to serve as a key mediator of Wnt signal transduction that is regulated through the following mechanism (for review see Gumbiner, 1995; Peifer, 1995; Mllier and M oon, 1996; Cadigan and Nusse, 1997). In the absence of a Wnt signal, β-catenin is confined to the plasma membrane, where it stably is associated with cadherin adhesion molecules. Cytoplasmic β-catenin levels are very low because free β-catenin is a target for GSK 3β-dependent phosphorylation and is degraded rapidly via the ubiquitin pathway. In the presence of a Wnt signal, GSK 3β phosphorylation of β-catenin is inhibited, free β-catenin is stabilized, accumulates in the cytoplasm, and is imported into the nucleus. β-Catenin can interact with HMG-box transcription factors of the TCF/Lef-1 family, leading to activation of specific target genes.

Despite the apparent simplicity of this signaling cascade, the mechanisms involved in the regulation of β-catenin are still rather obscure. For instance, it is still not known whether upstream components (Wnt, Frizzled, Dsh) affect GSK 3β activity or the accessibility of β-catenin to GSK 3β. The tumor suppressor gene product adenomatous polyposis coli (APC), which also binds directly to β-catenin, appears to be required to maintain low levels of β-catenin in mammalian cell lines (Munemitsu et al., 1995). However, experiments in embryonic systems are inconsistent with APC being only a negative regulator of β-catenin and suggest that it might, on the contrary, be an activator of the pathway (Rochelau et al., 1997; Vlemnickx et al., 1997). Finally, there is still no definitive evidence that regulation of the β-catenin level is the only important parameter in β-catenin signaling, or whether phosphorylation could affect directly its signaling activity. The apparent involvement of Axin in Wnt signal transduction, at a level close to GSK 3β and β-catenin, indicated that Axin might be at the heart of the process of β-catenin phosphorylation/degradation.

To investigate the mechanism by which Axin participates in the regulation of Wnt signal transduction, we undertook a study of the interaction of Axin with various components of the Wnt pathway. We also carried out a functional dissection of the role of different regions of components of the Wnt pathway. We also carried out a study of the interaction of Axin with various components of the Wnt pathway. We also carried out a study of the interaction of Axin with various components of the Wnt pathway. We also carried out a study of the interaction of Axin with various components of the Wnt pathway.
(HyClone Laboratories Inc.) in humidified 6% CO₂. Cells were transfected using a calcium phosphate mammalian cell transfection kit (5 Prime –3 Prime, Inc.). The next day cells were collected and lysed and analyzed for transient expression of transfected DNA.

**Immunoprecipitation (IP) and Western Blot**

For IP and Western blot analysis, 293 cells were washed with PBS, pH 7.2, and lysed in lysis buffer (150 mM NaCl, 5 mM EDTA, 50 mM NaF, 50 mM KH₂PO₄, 10 mM sodium molybdate, 20 mM Tris-HCl, pH 7.4, 5 μg/ml aprotinin, 5 μg/ml leupeptin, 0.6 mM DTT, 2 mM sodium orthovanadate, 0.2 mM PM SF, 1% Triton X-100). Aitter 20 min at 4°C with constant rotation, the lysate was centrifuged at 14,000g for 15 min and the supernatant was saved. Protein concentration was measured by the Lowry method (Lowry et al., 1951). For communoprecipitation (coIP) of Axin/PC/β-catenin/GSK3β complex, 150–300 μg (total protein) of cell lysate was incubated with 1–2 μg of an appropriate antibody in lysis buffer for 2 h at 4°C with constant rotation. 30 μl of protein A/G plus-agarose (Santa Cruz Biotechnology) was added and the incubation continued for an additional 1.5 h. Immunoprecipitates were pelleted and washed three times with lysis buffer. Immunoprecipitates were analyzed by SDS-PAGE GE (5% acrylamide for A/PC detection, 10% for detection of other proteins) and Western blot, using HRP-conjugated donkey anti-rabbit and sheep anti-mouse secondary antibodies (A mersham Life Science) and the chemiluminescence system (RENAISSANCE™; NEN Life Science Products). A approximately 10–20 μg of protein was used to detect expression of the various constructs.

**Direct Binding Assay**

Myc-APC constructs were produced with TNT coupled wheat germ extract system (Promega). These [35S]M et-labeled proteins were incubated with 2 μg of bacterially expressed S-tagged Axin fusion proteins in 500 μl of buffer (50 mM TRIS-HCl, pH 8.0, 150 mM NaCl, 2 mM EDTA, 5 mM DTT, 5 μg/ml aprotinin, 5 μg/ml leupeptin, 0.1 mM PM SF, 0.5% NP-40 at 37°C for 10 min and at 4°C for 10 min. The protein complexes were precipitated with 30 μl S-protein agarose (Novagen), washed three times with the same incubation buffer, and analyzed by SDS-PAGE GE (7.5% gel) and autoradiography.

**Mobility Shift Assay**

Lysates from cells transiently expressing VSV-G–tagged APC (VSV-A PC) and Axin constructs were used for IP with anti-VSV-G–P5D4 mAb. Immunoprecipitates were incubated with 1,000 U of λ-protein phosphatase (New England Biolabs Inc.) in 50 μl reaction buffer (50 mM TRIS-HCl, pH 7.8, 5 mM DTT, 2 mM M nCl₂, and 10 μg/ml BSA) at 30°C for 30 min. λ-Protein phosphatase-treated samples were separated by SDS-PAGE GE (5% gels) and VSV–APC was detected by immunoblot using the anti-Myc-APC constructs in bound and unbound fractions were analyzed by SDS-PAGE and immunoblot using the anti-Myc GE 10 ma.b.

**Cell Fractionation**

Early cleaving embryos were coinjected with 1 ng Myc-tagged Axin (Myc-Axin) and 3 ng β-galactosidase mRN A. At stage 9–10, 10 embryos were homogenized in 500 μl 250 mM sucrose, 110 mM potassium acetate, 10 mM H epes, pH 7.4, 2 mM magnesium acetate, 2 mM DTT, 1 mM EDTA supplemented with protease inhibitors. The homogenate was centrifuged for 5 min at 1,500g, and the low speed pellet was extracted in NP-40 buffer. The low speed supernatant was fractionated further by centrifugation for 30 min at 100,000g in a tabletop ultracentrifuge (TL-100; Beck- man Instruments Inc.) into a high speed pellet and supernatant. The fractions were analyzed for Myc-Axin and β-galactosidase by SDS-PAGE and immunoblot.

**Con A Precipitation**

FL A/PC or various mutant constructs mRN A were injected into 4–8-cell stage embryos A/PC or various Axin mutant mRN A. A mounts of mRN A injected were the following: β-galactosidase, 1 ng; FL A/PC (A x12-956), 1 ng; A x12-951, 0.5 ng; A x94-530, 0.5 ng; A x194-672, 0.25 ng; A x251-351, 1 ng; A x331-956, 0.5 ng; and A x331-956, 0.5 ng. Total amounts of injected mRN A were adjusted to 1.075 ng by addition of β-galactosidase mRN A. In some experiments (see Fig. 5 C), higher levels of β-catenin were tested using 0.75–1.5 ng mRN A. Embryos were extracted in NP-40 buffer at stage 9–10 and either directly analyzed by SDS-PAGE and immunoblot, or cleared from cadherin-bound β-catenin as follows: six embryos were extracted in 200 μl NP-40 buffer. 50 μl of Con A–agarose beads (75% slurry) were added, and the samples were incubated with constant mixing for 1–2 h. The beads were spun down and discarded and the supernatant was analyzed for β-catenin levels using an anti-HA tag rabbit antibody (Santa Cruz Biotech.), as well as for A/PC mutant levels using the 9E 10 ma.b.

**β-Catenin Stability**

HA–tagged β-catenin mRN A (75 pg) was coinjected with β-galactosidase mRN A (control) or various Aixin mutant mRN A. A mounts of mRN A injected were the following: β-galactosidase, 1 ng; FL A/PC (A x12-956), 1 ng; A x12-951, 0.5 ng; A x94-530, 0.5 ng; A x194-672, 0.25 ng; A x251-351, 1 ng; A x331-956, 0.5 ng; and A x331-956, 0.5 ng. Total amounts of injected mRN A were adjusted to 1.075 ng by addition of β-galactosidase mRN A.

In some experiments (see Fig. 5 C), higher levels of β-catenin were tested using 0.75–1.5 ng mRN A. Embryos were extracted in NP-40 buffer at stage 9–10 and either directly analyzed by SDS-PAGE and immunoblot, or cleared from cadherin-bound β-catenin as follows: six embryos were extracted in 200 μl NP-40 buffer. 50 μl of Con A–agarose beads (75% slurry) were added, and the samples were incubated with constant mixing for 1–2 h. The beads were spun down and discarded and the supernatant was analyzed for β-catenin levels using an anti-HA tag rabbit antibody (Santa Cruz Biotech.), as well as for A/PC mutant levels using the 9E 10 ma.b.

**Immunofluorescence (IF)**

Stage 9–11 embryos were fixed in 4% paraformaldehyde, 100 mM Hepes, pH 7.4, 100 mM NaCl for 1 h at room temperature, then in Dent’s fixative (20% DMSO, 80% methanol) overnight at -20°C. They were rinsed in 100 mM Tris-HCl, 100 mM NaCl, and embedded in 15%, then 25% fish gelatin, and 10-μm cryosections were prepared as described (Fagotto and Gumbiner, 1994; Fagotto, 1999). Sections were labeled with 9E 10 ma.b and anti-mouse Oregen green488 or A lexa488 secondary antibodies (Molecular Probes Inc.), the yolk counterstained with Eriochrome back, and nuclei with 4',6-diamidino-2-phenylindole (DA PI) as described (Fagotto, 1999). For double staining, sections from embryos coinjected with Myc–Axin and HA–tagged D sh (HA–D sh) mRN A’s (1 ng each) were stained simultaneously with anti-Myc rabbit pAb and anti-HA mAb 12CA5, followed by A lexa488 goat anti-rabbit and Cy3 donkey anti-mouse (Dianova) secondary antibodies.

For localization of Myc-Axin in cultured cells, HE.La cells cultured in DMEM were transfected with pCs2-Myc-Axin using LipofectAmine (GIBCO BRL). 36–48 h after transfection, cells were fixed in 4% paraformaldehyde/PBS, permeabilized with 0.05% Triton X-100, and labeled with anti-Myc 9E 10 ma.b and Cy3 goat anti-mouse (Dianova) secondary antibodies. Nuclei were counterstained with DAPI. Samples were observed with an A xiplan epifluorescence microscope (Zeiss) using standard fluorescein and Cy3 filters, and digital images were collected using a camera (768x576 3CCD color video; Sony).

**Electron Microscopy and Immunogold Labeling**

Preembedding labeling was performed as described (K urth, 1997; Fagotto, 1999). In brief, embryos expressing FL Myc-Axin or Myc-Axin x331-956-810 were fixed at stage 10 with 4% paraformaldehyde, 0.02% glutaraldehyde,
and 100 mM Hepes-NaOH. Labeling was performed by incubating 100 μm vibrome sections with the 9E10.2 mAb b and a Nanogold-coupled anti-mouse secondary antibody, followed by silver enhancement. The reaction produced electron dense aggregates with a diameter of ~20–60 nm (see Fig. 8, C–E). The sections were embedded in Spurr resin and ultrathin sections were prepared.

Postembedding labeling was performed on small blebs obtained from the wounds of injected embryos. These blebs contained a large number of Axin-expressing cells, and their ultrastructure was better preserved than in whole embryos (Kurth, 1997). Paraformaldehyde/glutaraldehyde-fixed samples were processed for Lowycryl embedding, ultrathin sectioning, and immunogold labeling (9E10.2 mAb and 15 nm gold-coupled protein G) according to standard procedures.

Results

Binding of Full-length and Mutant Forms of Axin to APC, GSK3β, and β-Catenin

When epitope-tagged full-length Axin (amino acids 12-956) was expressed in 293 cells, endogenous GSK3 β and β-catenin, as well as VSV-epitope tagged APC (VSV–A PC), could be coimmunoprecipitated (coIP) with Axin. A variety of mutant forms of Axin were used next for coIP and direct binding assays to further delimit the regions of Axin required for these interactions (Figs. 1 and 2 and data not shown) and to compare binding abilities with activity in functional assays (see below). The results are summarized in Fig. 3, which includes a schematic diagram of Axin, indicating the locations of the major binding sites for these proteins as well as PP2A binding and Axin self-binding (Hu et al., 1999).

The region of Axin corresponding to aa 561–630 of mAxin has been shown to contain a β-catenin binding site (Ikeda et al., 1998) and our results confirmed that all Axin mutants containing this region could coIP with endogenous β-catenin. However, several mutants that lacked this region but included the RGS domain of Axin (Ax12-531, Ax251-351), which eliminated APC binding region (aa 96–253) was not sufficient for coIP with VSV–A PC, whereas all but one of those lacking it failed to coIP with VSV–A PC (Figs. 2 D and 3). However, A x331-956 also was able to coIP with A PC, probably via β-catenin (Fig. 2 D). The second A PC binding region (aa 96–253) was not sufficient for coIP with VSV–A PC (Fig. 2 D, A x3251-351). Whether A x12-355 or A x12-167 could coIP with VSV–A PC could not be determined because expression of these mutant A xins resulted in a strong reduction in the level of VSV–A PC (Fig. 2 E and data not shown).

Axin Overexpression Induces Phosphorylation of APC In Vivo

When FL Axin and VSV–A PC were cotransfected into 293 cells, the electrophoretic mobility of VSV–A PC was reduced compared with control cells cotransfected with VSV–A PC plus pCS2 vector. A x12-810 and A x12-351 caused a similar mobility shift, whereas A x3231-351, A x12-355, and A x497-672, which lack either the GSK3 β or A PC binding site, did not (Fig. 2 E). It has been shown that phosphorylation of A PC by GSK3 β (Rubinfeld et al., 1996) can be stimulated by Axin in vitro (Hart et al., 1998).
Axin Sequences Necessary for Ventralization of Xenopus Embryos

We previously have shown that the ability of A xin to inhibit dorsal axis formation, when expressed in early Xeno-
opus embryos, is due to its inhibitory effect on the Wnt sig-
ning pathway (Zeng et al., 1997). Therefore, we used this
assy to delimit the sequences in A xin required for its neg-
ative effects on signaling through the Wnt pathway. 22 mu-
tant forms of A xin were expressed by mRNA injection on
the dorsal side of 4-cell stage embryos that were cultured
to the tadpole stage and examined for the extent of dorsal
axis formation (fraction of embryos ventralized and dorso-
terior index). The amount of injected mRNA s was sys-
tematically titrated to obtain comparable levels of expres-
sion for the various mutants. The results are summarized
in Fig. 3, the data are listed in Table I and examples are
shown in Fig. 4.

As we previously reported (Zeng et al., 1997), an in-
ternal deletion of the RGS domain (A x D 251-351) elimi-
nated the ability to ventralize and instead caused dorsal-
ization. Deletion of the GSK3 b and b-catenin binding sites
(A x D 352-631) also abolished ventralizing activity. A small
fragment containing the GSK3 b and b-catenin binding sites
(A x D 497-672) was insufficient to ventralize the em-
byo, although similar A xin fragments were able to pro-
mote phosphorylation of b-catenin in vitro (Ikeda et al.,
1998). A fragment containing only the RGS domain
(A x D 194-353) was also ineffective.

Successive truncation from the NH 2 terminus of A xin
confirmed the importance of the RGS domain for ventral-
ization. Whereas removal of the first 193 aa had no signifi-
cant effect, further truncation to aa 331 eliminated ventral-
izing activity and resulted in dorsalizing activity, similar to
the internal RGS deletion (Fig. 4, D and F). Truncation at
aa 531, removing both the RGS domain and GSK3 b bind-
ning region, eliminated all activity (Fig. 4 C) as did trunca-
ton to aa 810 (A x 810-956).

Mutant A xins with NH 2-termini at aa 194 were sub-
jected to COOH-terminal truncation to examine the im-
portance of the DIX, PP2A binding, b-catenin binding,
and GSK3 b binding domains. Removal of the DIX do-
main (A x D 194-810) had little if any effect, whereas removal
of the DIX and PP2A binding domains (A x D 194-672)
caused an increase in ventralizing activity (Fig. 4 B). This
observation is consistent with the hypothesis that the bind-
ing of PP2A to the A xin complex may negatively regulate
the phosphorylation of b-catenin by GSK3 b (Hsu et al.,
1999). Further truncation to aa 531, removing the b-cate-
nin binding site, abolished ventralizing activity, and in-
stead resulted in some dorsalizing activity (i.e., shorter
axis, larger head, and circular or double cement gland; Fig.
4 E). When injected at high concentrations, the other A xin
mutants lacking the NH 2-terminal and the COOH-termi-
nal regions (A x D 194-672 and A x D 194-810) also showed dor-
salizing activity, as discussed below.

When the NH 2 terminus of A xin was left intact, trunca-

To test whether this A xin-induced mobility shift was due
to phosphorylation, the immunoprecipitated proteins were
treated with â-protein phosphatase before immunoblot
analysis with anti-VSV. This treatment eliminated the mo-

Figure 2. A xin binds to A PC and induces A PC phosphorylation
in vivo. (A) Diagram showing the regions of human A PC in-
cluded in three A PC constructs. (B) Direct in vitro binding
of A PC25 to A xin fragments containing the RGS domain. Sulfur-
35-labeled A PC25 protein was incubated with S-tagged fusion
proteins containing the indicated regions of A xin. A fter S-pro-
tein agarose IP, bound A PC25 was detected by SDS-PAGE and
autoradiography. Coomassie blue staining of the A xin fusion
proteins is shown at the bottom. (C) In vitro binding with NH 2-
terminal A xin fragments reveals a second A PC-binding site up-
stream of the RGS domain. Binding of A PC2 and A PC25 re-
quired the RGS domain (A x 194-353 and A x 194-480). However, FL
A PC also interacted with A x 194-253, and hA PC2 bound A x 196-
253 very strongly. Interaction of Sulfur-35-labeled FL A PC, A PC2,
A PC21 and A PC25 with S-tagged fusion proteins was de-
termined as in B. (D) CoIP of VSV–APC with FL A xin and sev-
eral mutant forms of A xin. 293 cells were cotransfected with
VSV-G–tagged Xenopus A PC (VSV–APC) and the indicated
A xin constructs. The cell lysates were immunoprecipitated with
anti-Myc and probed with anti-VSV-G antibody. Failure to
detect interaction with A x 12-355 was due to the very low levels
of VSV–APC observed when coexpressed with this particular con-
struct (E). (E and F) A xin-dependent phosphorylation of A PC.
(E) A xin-induced mobility shift of VSV–A PC. Total lysates from
cotransfected cells were analyzed by Western blot using an anti-
VSV mAb. FL A xin and mutants A x 12-810, 12-600, and 12-531
induced a mobility shift, whereas A x 12-351, 12-155, and A x 12-355,
and A x 497-622 did not. (F) Phosphatase treatment eliminated the
mobility shift. Cotransfected cell lysates were IP with anti-VSV
mAb, and the products were analyzed by SDS-PAGE and West-
ern blot before (left) or after (right) incubation with â-protein
phosphatase.
tion of the COOH terminus to remove the DIX domain, or both the DIX and PP2A domains, caused only a slight reduction, if any, in the ability to ventralize. Surprisingly, there was no further reduction in activity when the region including the β-catenin binding site was truncated (Ax12-531) or removed by an internal deletion (AxD531-810).

Unlike the mutants with NH2-termini truncated at aa 194, no dominant negative effect (dorsalization) was seen when high concentrations were injected (Table II). Further truncation, removing the GSK3β site, eliminated all activity (Ax12-355). Internal deletion of only the RGS domain, in the context of COOH-terminal truncations (mutant Ax12-810Δ251-351, Ax12-672Δ251-351, and Ax12-531Δ251-351), also eliminated ventralizing activity and instead cause weak dorsalizing activity.

Dorsalization

In contrast to the ability of Axin and other inhibitors of Wnt signaling (e.g., GSK3β) to ventralize when injected dorsally, factors that stimulate this pathway (e.g., certain Wnts, Dsh, dnGSK3β, or β-catenin) have dorsalizing activity; when injected dorsally, they can hyperdorsalize (i.e., they induce formation of a larger head, large or multiple cement glands, shorter axis, and double anterior axis). However, their activity is best seen in ventral injections, where they can induce a secondary axis (Miller and Moon, 1996; Fagotto et al., 1997; Fagotto, 1999). We previously showed that a mutant Axin lacking the RGS domain (AxD251-351) behaved as such a dorsalizing factor. This activity could be competed by coexpression of FL Axin, supporting the conclusion that it was due to a dominant negative effect (Zeng et al., 1997). To identify the domains of Axin required for this activity, several additional mutant forms of Axin were also injected into the ventral side of the embryo to assay their ability to induce axis duplication.

Sequences upstream from the RGS domain were not required, as an NH2-terminal truncation at aa 331 (Ax331-956) induced axis duplication as efficiently as the RGS deletion (Figs. 3 and 4F, Table II). However, the GSK3β binding site was required as truncation at aa 531 abolished the effect (Ax531-956). The COOH-terminal sequences were also important; in the presence of the RGS deletion, COOH-terminal truncation at aa 810, 672, or 530 strongly reduced the dorsalizing activity so that axis duplication...
Effects of Axin and Axin Mutants on β-Catenin levels

Modulation of the Wnt pathway has a striking effect on β-catenin levels; in the absence of a Wnt signal, constitutively active GSK3β phosphorylates β-catenin and causes its rapid turnover, whereas Wnt signaling induces stabilization of β-catenin. Therefore, we tested the effect of Axin on β-catenin levels in X. tropicalis embryos by coexpressing HA-tagged β-catenin with FL or mutant forms of Axin, or with β-galactosidase as a control. Low amounts of HA-tagged β-catenin mRNA were used to mimic the behavior of endogenous β-catenin. At the late blastula stage, when endogenous β-catenin signaling peaks (Lemaire et al., 1995; Schneider et al., 1996), the levels of HA-tagged β-catenin in embryo extracts were analyzed. The membrane (cadherin-bound) pool of β-catenin is known to be very stable (Kofron et al., 1997) and changes in β-catenin levels by the Wnt signaling pathway affect mostly the unbound, soluble pool (Riggleman et al., 1990; Peifer et al., 1994; Pai et al., 1997). Removing the cadherin-bound pool of β-catenin by Con A precipitation made it possible to obtain samples enriched in soluble β-catenin, allowing the effect of Axin on β-catenin levels to be analyzed more accurately. As shown in Fig. 5 B, FL Axin caused a dramatic decrease in exogenous β-catenin. Several mutant Axin constructs were coexpressed similarly; mutants with ventralizing activity (A x12-531 and A x194-672) also proved to be effective in reducing β-catenin levels. A mutant lacking ventralizing activity (A x531-956) had no effect on β-catenin levels. On the other hand, mutants with strong dominant negative activity (A x1251-351 and A x331-956) induced a clear increase in β-catenin levels. Unexpectedly, mutant A x194-531, which failed to ventralize over a wide range of concentrations, but instead showed some dorsalizing activity, caused a strong decrease in β-catenin levels.

The activity of the dominant negative mutant A xΔ251-351 was also tested on the ventral side, where the β-catenin degradation machinery is maximally active. Under these conditions, stabilization of β-catenin by A xΔ251-351 could be observed even in total extracts (Fig. 5 C). NH2 terminally deleted β-catenin (hemagglutinin epitope-tagged Δ-NH2 terminus β-catenin; Funayama et al., 1995), which lacks the GSK3β-dependent phosphorylation site (Munemitsu et al., 1996; Yost et al., 1996), was found to be insensitive to Axin overexpression (Fig. 5 D), suggesting that Axin-induced destabilization of β-catenin requires phosphorylation by GSK3β.

Intracellular Distribution of Axin

As the subcellular localization of endogenous Axin is so far unknown, we examined the distribution of the ectopically expressed Myc-tagged Axin in X. tropicalis embryos. As shown in Fig. 6, FL Myc-Axin exhibited a striking and unusual pattern. The signal was mostly concentrated in very bright spots, which were found singly or in clusters of variable size, mainly, but not exclusively, at the cell periphery.
Table I. Effect of Axin Mutants on Axis Development: Dorsal Injections

| Mutant          | Amount mRNA injected | Total injected embryos | Percent ventralized | Average DAI | Ventralization | No. exp
|-----------------|----------------------|------------------------|---------------------|-------------|----------------|---------|
|                 | ng                    |                        |                     |             |                |         |
| FL (12-956)     | 2 × 0.13              | 28                     | 46                  | 3.6         | +/−            | 2       |
| FL (12-956)     | 2 × 0.25              | 57                     | 70                  | 2.5         | +              | 2       |
| FL (12-956)     | 2 × 2                 | 103                    | 94                  | 1.0         | ++ +           | 6       |
| Δ251-351        | 2 × 1–2               | 60                     | 4                   | ?           | dorsalized     | 5       |
| Δ352-631        | 2 × 1                 | 61                     | 26                  | 4.3         | −              | 4       |
| Δ531-810        | 2 × 0.5–2             | 63                     | 89                  | 1.6         | + +            | 5       |
| 194-956         | 2 × 0.5–1             | 107                    | 91                  | 1.6         | + +            | 5       |
| 331-956         | 2 × 0.25              | 40                     | 10                  | 4.9         | −              | 3       |
| 331-956         | 2 × 0.5–1             | 63                     | 0                   | ?           | dorsalized     | 3       |
| 531-956         | 2 × 0.5–1.5           | 107                    | 11                  | 4.6         | −              | 6       |
| 810-956         | 2 × 0.5–1             | 50                     | 4                   | 4.8         | −              | 4       |
| 194-810         | 2 × 0.5–1             | 98                     | 84                  | 2.1         | + + (*)        | 5       |
| 194-672         | 2 × 0.125–0.5         | 168                    | 94                  | 0.6         | + + + (*)      | 7       |
| 194-531         | 2 × 0.125–1           | 204                    | 12                  | ?           | dorsalized     | 10      |
| 194-353         | 2 × 1–2               | 47                     | 11                  | 4.6         | −              | 3       |
| 12-810          | 2 × 0.5–2             | 104                    | 86                  | 1.8         | + +            | 5       |
| 12-672          | 2 × 1                 | 63                     | 87                  | 1.6         | + +            | 3       |
| 12-531          | 2 × 0.5–2             | 149                    | 85                  | 1.9         | + +            | 7       |
| 12-355          | 2 × 0.5–1             | 105                    | 11                  | 4.6         | −              | 6       |
| 12-167          | 2 × 0.5–2             | 63                     | 16                  | 4.5         | −              | 4       |
| 531-810         | 2 × 0.75–2            | 42                     | 12                  | 4.4         | −              | 3       |
| 12-810Δ251-351  | 2 × 1                 | 34                     | 0                   | ?           | dorsalized     | 3       |
| 12-762Δ251-351  | 2 × 1–2               | 54                     | 2                   | 4.8         | −              | 3       |
| 12-531Δ251-351  | 2 × 0.25–1            | 27                     | 0                   | 5.0         | −              | 2       |
| 497-672         | 2 × 0.5–1             | 78                     | 9                   | 4.8         | (d)            | 4       |
| 403-600         | 2 × 0.25–1            | 119                    | 8                   | 4.9         | (d)            | 7       |

†Because expression levels varied considerably between different constructs, mutant Axin mRNAs were systematically titrated and expression levels were compared by Western blot, using the levels obtained with 2 × 2 ng FL Axin mRNA as a reference.

§Percent ventralization indicates the frequency of embryos with a DAI  < 4.

‡Because expression levels varied considerably between different constructs, mutant Axin mRNAs were systematically titrated and expression levels were compared by Western blot, using the levels obtained with 2 × 2 ng FL Axin mRNA as a reference. Consistent with the IF data, Axin was found to be distributed unambiguously by EM, probably because it was generally too weak (Fig. 6 A, IF). On the other hand, Fig. 7 D and E, shows very large dense Myc-positive areas, where vesicles were tightly packed and consequently the dense cytoplasm appeared less prominent. Fig. 7 B shows a cluster of intermediate size and vesicle density. Plasma membrane localization of FL A xin could not be detected unambiguously by EM, probably because it was generally too weak (Fig. 6 A, IF). However, strong plasma membrane staining could be observed for the mutant A xin A α 531-810 that is consistent with IF results (Fig. 3).
cause binding to Con A of all Axin deletion mutants tested (Fig. 8 D) strictly correlated with plasma membrane localization (as detected by IF, see below).

**Colocalization of Dsh with Axin**

The punctate distribution of Axin strongly was reminiscent of the localization pattern of ectopically expressed Dsh (Yang-Snyder et al., 1996; Axelrod et al., 1998) (the distribution of endogenous Dsh in Xenopus is not known). Thus, we compared the localization of coexpressed Myc-tagged Axin and HA-Dsh by double IF. We observed a very good colocalization of these two proteins (Fig. 6, C and C′): HA-Dsh was detected at all sites positive for Myc-Axin (arrowshead), although some other spots were positive for Dsh but negative for Axin (arrows). The Myc-Axin pattern in these embryos was indistinguishable from the pattern observed in the absence of exogenous Dsh, suggesting that Dsh does not influence Axin localization. In contrast, Myc-Axin overexpression clearly affected HA-Dsh distribution: when HA-Dsh was expressed alone, it localized exclusively in single cytoplasmic spots, or small clusters of spots, distributed throughout the cell (Fig. 6 D). No membrane staining was observed. However, when coexpressed HA-Dsh and Myc-Axin colocalized in a pattern typical for overexpressed Axin (enrichment of spots at the cell periphery, presence of large clusters, and plasma membrane staining). These results suggest that Dsh may bind, directly or indirectly, to the Axin complex.

**Sequences Required for Axin Localization**

To examine the sequences in Axin that target it to its specific locations, and the functional significance of this localization, we examined the intracellular distribution of the same mutant forms used above. Internal deletion of the RGS domain had little or no effect on localization; the mutant protein localizing primarily in the spots and less at the plasma membrane (Fig. 3 and Fig. 9, B and C). Deletion of the GSK3β and β-catenin binding sites (AxD352-631) also had no effect on localization to the spots, but eliminated the membrane staining (Fig. 9, H and I). Deletion of the COOH-terminal 146-aa resulted in localization mainly at the membrane, with little or no labeling of the spots, e.g., AxD194-956 (not shown) AxD12-810 (Fig. 9 F), Ax12-531 (Fig. 9 G), and AxD12-351 (Fig. 9 J).

Forms of Axin lacking the NH2-terminal half displayed a mostly diffuse cytoplasmic localization, e.g., AxD497-672 (Fig. 9 D). When the APC and GSK3β-binding domains were left intact, there seemed to be some enrichment at the cell periphery (Fig. 9 E, AxD194-956), although it was difficult to assess the extent of membrane enrichment, because of the high cytoplasmic signal.

Thus, the NH2-terminus of Axin appears to be required for the characteristic pattern of localization, both in the cytoplasmic spots and at the membrane. The presence of the normal COOH-terminus tends to cause localization to the spots, although it is not absolutely required for this. The COOH-terminus, which includes a dimerization domain, might bind to endogenous Axin or to other cellular components. The APC and GSK3β-binding sites appear to have a weaker effect on localization at the membrane. The membrane localization of mutants containing the NH2-terminus correlates very well with Con A binding (Fig. 8 C). For instance, AxD12-531 binds very efficiently to Con A, whereas AxD351-956 does not bind at all. However, localization to the spots appears to depend on a different mechanism, only a small fraction of FL Axin and an even smaller fraction of AxD351-630 (found mostly in spots) bind to Con A (Fig. 8 C and data not shown).

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**Table II. Effect of Axin Mutants on Axis Development: Ventral Injections**

| Mutant                  | Amount mRNA injected | Total injected embryos | Percent DA | Dorsalization | cDA | pDA | vDA | N | No. exp |
|-------------------------|----------------------|------------------------|------------|---------------|-----|-----|-----|---|--------|
| FL (12-956)             | 1–4                  | 58                     | 0          | –             | 0   | 0   | 0   | 58 | 4      |
| Δ251-351                | 1–2                  | 148                    | 80         | + + +         | 54  | 65  | 12  | 17 | 9      |
| Δ352-631                | 1                    | 39                     | 0          | –             | 0   | 0   | 0   | 39 | 3      |
| 331-956                 | 0.25                 | 59                     | 19         | +             | 0   | 11  | 0   | 48 | 3      |
| 331-956                 | 1                    | 129                    | 87         | + + +         | 83  | 29  | 3   | 14 | 4      |
| 531-956                 | 0.5–1.5              | 62                     | 0          | –             | 0   | 0   | 0   | 62 | 3      |
| 194-531                 | 0.5                  | 97                     | 24         | +             | 9   | 14  | 36  | 38 | 4      |
| 194-353                 | 0.5–1                | 34                     | 0          | –             | 0   | 0   | 4   | 30 | 2      |
| 12-672                  | 1                    | 49                     | 0          | –             | 0   | 0   | 0   | 49 | 2      |
| 531-810                 | 0.75–1               | 34                     | 0          | –             | 0   | 0   | 0   | 34 | 2      |
| 12-810Δ251-351          | 0.25*                | 23                     | 0          | –             | 0   | 0   | 2   | 21 | 2      |
| 12-810Δ251-351          | 0.5–2                | 149                    | 56         | ++            | 15  | 69  | 6   | 59 | 9      |
| 12-762Δ251-351          | 0.25–2               | 168                    | 1          | –             | 0   | 1   | 10  | 157| 9      |
| 12-531Δ251-351          | 0.25*                | 28                     | 0          | –             | 0   | 0   | 0   | 28 | 2      |
| 12-531Δ251-351          | 0.5–2                | 53                     | 30         | +             | 4   | 12  | 6   | 31 | 4      |
| 497-672                 | 0.5                  | 45                     | 0          | –             | 0   | 0   | 0   | 45 | 2      |
| 403-600                 | 0.5–1                | 91                     | 3          | –             | 0   | 3   | 0   | 88 | 4      |

*Expression levels equivalent to 1–2 ng FL Axin mRNA.

1Expression levels higher than for FL and 12-956Δ251-351 Aixin.

DA, duplicated axis; percent DA, all duplicated axis (cDA); pDA, partial DA; vDA, vestigial DA; N, normal embryos; and No. Exp, number of experiments.
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Axin has been shown to negatively regulate signaling through components of the Wnt pathway. Coinjection experiments in Xenopus embryos previously suggested that it acts downstream of GSK 3β and upstream of β-catenin. Subsequent studies have shown that Axin is part of a complex including these two proteins as well as APC and that it promotes the phosphorylation of β-catenin by GSK 3β and its subsequent degradation (Hart et al., 1998; Ikeda et al., 1998; Itoh et al., 1998; Kishida et al., 1998; Sakanaka et al., 1998). The aims of the experiments reported here were to understand the relationship between Axin's ability to bind to these and other proteins and its capacity to function in the regulation of this pathway. To this end, we have examined a series of Axin mutants for their ability to (1) bind to APC, GSK 3β, and β-catenin; (2) ventralize or dorsalize Xenopus embryos, an established assay for effects on β-catenin signaling; and (3) alter the stability of β-catenin expressed from coinjected mRNA. In addition, we have examined the intracellular localization of FL Axin and a series of Axin mutants.

**Interaction of Axin with Other Components of the Wnt Signaling Pathway**

Through direct binding in vitro and coIP from mammalian cell extracts, we have confirmed that Axin forms a complex with APC, GSK 3β, and β-catenin, and we have further delimited some of the binding sites for these proteins. Based on coIP, the region of mAxin required for interaction with GSK 3β lies between aa 497 and 531. The COOH-terminal boundary of the minimal binding region appears to lie between aa 526 and 531. Whereas Itoh et al. (1998) did not detect coIP of GSK 3β with Axin 12-526, we detected weak interaction of GSK 3β with some Axin mutants terminating at aa 531. We confirmed that the RGS domain (aa 220–340) includes a major binding site for APC and interacts with the 20-aa repeat region of APC. Furthermore, we identified a second region of Axin, between aa 96–253, that can bind directly to the NH2-terminal region of APC containing the Armadillo and 15-aa repeat. This region of Axin was neither necessary nor sufficient for coIP with VSV-G–tagged APC, suggesting that it plays a secondary role to the RGS domain in vivo.

We observed good agreement between the presence of the direct binding site for GSK 3β (Ikeda et al., 1998) and the ability of Axin mutants to coIP with GSK 3β (Fig. 4). Axin mutants lacking this region, some of which were able to coIP with APC and/or β-catenin, failed to coIP with GSK 3β, suggesting that GSK 3β must bind directly to Axin to join the complex. However, several forms of Axin that lacked the direct binding site for either APC or β-catenin were able to coIP with both of these proteins. A PC was found to coIP not only with all forms of Axin containing the RGS domain, but also with Axin 12-531 and Axin 194-672, which lacks any direct binding site for APC. Similarly, β-catenin could coIP not only with all Axin mutants containing Aα 600–622, but also with Axin 12-531 and Axin 194-672. Both of these discrepancies likely are due to the binding of APC and β-catenin to bind each other (Rubinfeld et al., 1993; Su et al., 1993). Since each of these three components can interact directly with the other two, we suggest that they form in vivo a triangular complex (Fig. 10).

The GSK 3β binding domain was dispensable for direct binding to APC and β-catenin, but required for indirect binding to β-catenin (presumably via APC). Indeed, a small fragment of Axin containing only the RGS domain (A x194-353), while able to coIP with APC, did not coIP...
with β-catenin. Conversely, A x531-956 was found to colP
with β-catenin but not with A PC. We also found that A xin
induced a mobility shift in A PC that appeared to be due to
phosphorylation. This activity required the GSK3
β-binding domain as well as the A PC-binding region, suggest-
ing that GSK3 β is responsible for this modification. It previ-
ously has been shown that GSK3 β can phosphorylate
A PC in vitro (Rubinfeld et al., 1996), that A xin promotes
this event (Hart et al., 1998), and that this phosphorylation
enhances the ability of A PC to bind to β-catenin (Rubin-
feld et al., 1996). Our observations argue that A xin per-
forms a similar function in vivo.

Axin Sequences Required to Influence Axis Formation
and Regulate β-Catenin Levels in Frog Embryos

In general, we found a good correlation between the abil-
ity of A xin mutants to ventralize frog embryos and to
lower the levels of coinjected HA-tagged β-catenin, pre-
sumably by promoting its degradation. The RGS domain
and GSK3 β binding site were both required, although not
sufficient, for A xin activity. In addition, either the β-cate-
nin binding site or the NH2-terminal region upstream of
the RGS domain (but not necessarily both) was required.

The activity of mutant forms of A xin lacking the β-catenin
binding site (e.g., A x12-531 and A x531-810) is consistent
with the observation that such forms can colP with β-cate-
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Electron microscopic localization of Myc-Axin. (A–C) EM localization of FL Myc-Axin by on-section staining on Lowycryl sections. (A) Low magnification view of an Axin-positive cluster detected by indirect IF on a thin section. Axin expression in this cell is relatively low and individual spots can be resolved (arrows). (B) Low magnification EM image of a cluster of gold particles (15 nm) labeling an area of dense cytoplasm (arrows) containing numerous vesicles (asterisks). Note that outside the cluster the surrounding cytoplasm is devoid of gold particles. (C) High magnification view of portion of a less dense cluster. The cluster is composed of small groups of gold particles decorating electron dense cytoplasm (arrows) associated with a few vesicles (asterisks). Each small group probably corresponds to a single “spot” observed by IF (A and Fig. 6, A and B). Note that the membranes surrounding the vesicular structures appear much less contrasted in B and C compared with E. This is due to the difference in the methods used (low contrast Lowycryl sections in B and high contrast conventional Spurr sections in D). (D and E) EM localization of Myc-Axin by preembedding Nanogold labeling and silver enhancement. (D) Low magnification view of a Myc-Axin positive area (arrow) in a high expressing cell. Single gold aggregates found both in the cytoplasm and in the nucleus (n) represent background that is higher with this preembedding technique. The arrowhead points to the plasma membrane that shows no significant staining in this cell. (E) High magnification view of a similar area, packed with vesicles of variable size (~50-200 nm) embedded in electron dense cytoplasm. The irregular shape of the gold/silver particles is due to the silver enhancement method. Insert shows enlarged view (2×) of the outlined area, with tightly-packed vesicles (asterisks) and electron dense cytoplasm (arrow). Note that gold labeling tends to be somewhat excluded from the areas particularly packed with vesicles. This could be due to limited diffusion of Nanogold in these preparations. (F) Nanogold localization of Myc-Axin at the plasma membrane. Because FL Axin localization at the membrane is weak (D and Fig. 7 A), a mutant Axin, A xΔ331-810 was used in this experiment. In this case, the plasma membrane is heavily decorated with gold/silver particles (arrowheads). m, mitochondrion; p, pigment granules; and yp, yolk platelets. Bars: (A) 2 μm; (B) 1 mm; (C) 0.2 μm; (D) 1 μm; (E) 0.5 μm; (F) 1 μm.

Experiments (Fig. 5 C), we found that FL Axin was able to downregulate even very large amounts of co injected β-catenin. However, under these conditions several Axin mutants gave results that were inconsistent with their effects at more physiological β-catenin levels and with their activity on axis induction. In particular, both dominant negative mutants A xΔ251-351 and A x331-956 failed to stabilize β-catenin expressed at high levels, but rather caused some destabilization (not shown). Thus, Axin is capable of stimulating β-catenin degradation independently of the RGS domain, but only provided high levels of free β-catenin and/or absence of APC. Consistent with this observation, the Axin-like protein Conductin/Axl/Axin-2 appears to behave similarly. Indeed, a ARGS Conductin construct could downregulate β-catenin levels in SW 480 cells, yet acted as a dominant negative (i.e., increased β-catenin levels) in Neuro2A cells, which have low levels of endogenous β-catenin and FL APC (Behrens et al., 1998). It is likely that high levels of β-catenin are flooding the system, bypassing normal regulatory mechanisms. Nevertheless, it is also possible that two different mechanisms exist, one Apc-dependent, and one Apc-independent, active at low and at high β-catenin concentrations, respectively (see below).

Surprisingly, in SW 480 cells, truncation of the NH2 terminus, including the RGS domain, increased the activity of Axin that led to the proposal that the RGS domain may repress Axin activity in the absence of APC (Hart et al., 1998). However, this mutant construct could not discriminate between a role of the RGS domain itself and an effect of upstream sequences. In contrast, ARGS Conductin, a mutant with an internally deleted RGS domain and an intact NH2 terminus, showed weaker activity than FL Conductin in SW 480 cells, arguing that the RGS domain is not responsible for the apparent repression reported by Hart et al. (1998). In fact, SW 480 cells are not null for APC, but still contain an NH2-terminal fragment, which can bind β-catenin (Polakis, 1995), and can also interact with the NH2 terminus of Axin upstream of the RGS domain (our data). Thus, it is conceivable that the truncated APC may interfere with Axin activity.

In embryos, deletion of the RGS domain caused a strong dominant negative effect. The self-binding region (including the DIX domain) appeared to play a role in this activity, because deletion of this region from A xΔ251-351 substantially reduced activity. As the COOH-terminal 100 aa of Axin can mediate multimerization, the strong dominant negative forms of Axin may act by binding to endoge-
nous Axin. However, forms of Axin lacking the DIX domain also showed weak or moderate dorsalizing activity (Ax12-810Δ251-351 and Ax12-531Δ251-351), suggesting that dominant negative activity may be generated in more than one way. One possibility is that these forms of Axin bind to GSK3β but not to APC, thus, interfering with the formation of the complete complex. Also, two mutants lacking both the NH2 and COOH termini (Ax-531-956 and Ax194-353) or the NH2-terminal domain showed no (Ax-531-956 and Ax194-353) or very weak binding (Ax194-956).

The NH2- and COOH-terminal portions of Axin were not required for its ventralizing activity in our assays, as also observed by Itoh et al. (1998), although they may modulate its function. Deletion of the DIX domain had little or no effect on activity. Deletion of the PP2A binding region caused some increase in activity of the forms of Axin that initiated at aa 194, although this difference was not apparent when the NH2 terminus of Axin was left intact. We have hypothesized that the binding of PP2A to the Axin complex might counteract the phosphorylation of β-catenin by GSK3β (Hsu et al., 1999) that could account for the increased ventralization activity in the absence of this domain.

The Intracellular Localization of FL and Mutant Forms of Axin

FL Myc-Axin expressed in Xenopus embryos was found primarily in characteristic spots, singly or in clusters of variable size. Ultrastructural analyses indicated that the spotty distribution is not merely due to aggregation of an overexpressed protein, but corresponds to particular (though as yet ill-defined) subcellular structures, consisting of clustered vesicles associated with dense cytoplasm. Several arguments suggest that endogenous Axin has a similar distribution including: (1) identical spots were observed over a wide range of Axin expression levels.

HA-Dsh (Fig. 6) accumulated in similar spots in the absence of overexpressed Axin. (3) Myc-Axin colocalized with HA-Dsh (Fig. 6). However, the formation of large clusters of spots very likely is due to Axin overexpression, as it was not observed for HA-Dsh in the absence of Axin expression. However, it may reveal the ability of Axin to act as a scaffold through multiple interactions with other cytoplasmic proteins. A small, variable fraction of Myc-Axin was also associated with the plasma membrane, and Con A-binding showed that Axin interacts with a cell surface glycoprotein. The NH2 terminus is sufficient for membrane targeting, although some other internal sequences may also confer weaker binding. Interestingly, the sequences of Axin crucial for its subcellular localization (i.e., the NH2 and COOH termini) do not bind any of the core components related to its activity (APC, β-catenin, and GSK3β), and are apparently dispensable, at least under conditions of overexpression (Fig. 10). Clearly, additional molecular interactions must take place at both ends of the molecules.

The occurrence of two well-defined locations for Myc-Axin may reflect the existence of two functionally distinct pools, possibly an active and an inactive one. Ectopically expressed Dsh shows a similar dual localization at spots/membrane, which can be manipulated by overexpression of Wnt/Frizzled, and that may correspond to different functional states (Yanagawa et al., 1995; Axelrod et al., 1996; Steitz et al., 1996; Yang-Snyder et al., 1996). However, which would be the active and inactive sites remains unclear. The comparison of various Axin mutants did not reveal any simple correlation between their activity in functional assays and their intracellular distribution, although all active mutants can to some extent localize at the plasma membrane. However, it is quite possible that localization per se is not required for activity, but that regulation is achieved by sequestering various components of the signaling pathway in different compartments of the cell. It is then easy to conceive that overexpression may bypass such regulation and allow Axin/Axin mutants to

Figure 8. Cell fractionation of Myc-Axin in Xenopus embryos: sedimentability and Con A binding. (A) Diagram of Axin molecule. (B) Myc-Axin is associated with a sedimentable fraction. Homogenates of late blastula embryos were fractionated into a low speed sedimentable fraction (P, pellet), a high speed sedimentable fraction (M, membranes), and a high speed supernatant (S, soluble fraction) as described in Materials and Methods. β-Galactosidase was coexpressed and used as a control for soluble cytosolic proteins. Unlike β-galactosidase, Myc-Axin was sedimentable under these conditions. (C) Myc-Axin is fully extractable in NP-40. Embryos expressing Myc-Axin were extracted in NP-40-containing buffer (sol). The insoluble pellet was reextracted in the presence of SDS (insol, NP-40-insoluble). (D) A pool of Axin is associated with a membrane glycoprotein, and this association requires the NH2-terminal domain. Myc-tagged FL Axin and various Axin mutant constructs were expressed in embryos, and NP-40 extracts were fractionated using Con A beads. Bound fractions (B) were four times concentrated relative to unbound fractions (U). FL Axin showed significant association with Con A beads that indicates a stable interaction with a membrane glycoprotein. Stronger binding was observed for the NH2-terminal fragments Ax12-531. On the other hand, constructs lacking the NH2-terminal domain showed no (Ax-531-956 and Ax194-353) or very weak binding (Ax194-956).
teract with other components of the complex independently of upstream signals.

Our observations provide some hints for a role of Axin localization. For instance, the concentration-dependent dual activity of A x194-672 and A x194-810 may be related to their diffuse distribution; at high concentrations, they could act as dominant-negatives by affecting the balance between various endogenous components otherwise strictly compartmentalized. This dual activity was also found for the Axin-like protein Axil/Conductin/Axin-2 that has a similar diffuse distribution (Zhang, T., F. Fagotto, and F. Costantini, manuscript in preparation), but was never observed with Axin constructs showing a well-defined localization (spots and/or plasma membrane).

Conclusions and Models

Our functional data emphasize the essential role of the RGS domain for Axin activity (Fig. 10). They demonstrate that binding to GSK3β and to β-catenin, which was reported to stimulate β-catenin phosphorylation in vitro (Hart et al., 1998; Ikeda et al., 1998), is not sufficient in vivo either for β-catenin degradation or for inhibition of its signaling. Although the RGS domain may interact with other yet uncharacterized molecules, its importance most likely resides in its ability to bind APC, thus inducing the formation of a trimeric complex Axin•β-catenin•APC (Fig. 10). Note that the RGS domain of Axin is significantly diverged from the sequences of bona fide RGS proteins (Tesmer et al., 1997; Zeng et al., 1997), and does not appear to bind to G-proteins (our unpublished data) or to have RGS activity (Ma et al., 1998). Apparently, it has diverged toward other interactions and functions.

The apparent discrepancy with other data that suggests, under certain conditions, Axin can function without the RGS domain (Hart et al., 1998), may be best reconciled by...
postulating two mechanisms, active at different levels of free β-catenin: When β-catenin levels are low, β-catenin degradation would depend primarily of an Axin•β-catenin•APC complex, whereas when β-catenin levels are high, β-catenin•Axin complexes may form and function in the absence of a APC. Both mechanisms may be physiologically important: in the absence of Wnt signal, very low levels of free β-catenin might be maintained by the combined action of Axin and A PC. However, after a burst of Wnt signal, excess β-catenin would be first downregulated by an APC-independent coarsome mechanism, before fine tuning by a APC could eventually restore normal levels.

The occurrence of an Axin-based complex has further potential implications on the regulation of the pathway by Wnt. It had been assumed that Wnt caused β-catenin stabilization by inhibiting GSK3β activity (for review see Miller and Moon, 1996). However, GSK3β inhibition could hardly account for the specificity of the pathway, considering the many other substrates and pleiotropic functions of GSK3β. We now know that binding of GSK3β to Axin is required for phosphorylation of Axin, APC, and β-catenin, and ultimately for activity of the complex (Hart et al., 1998; Ikeda et al., 1998; this paper; Jho, E.-H., manuscript submitted for publication). Therefore, inhibition of Axin-GSK3β binding, or in fact any other interactions within the complex, could be a far more specific way to regulate this pathway. This might be precisely the function of Dsh. In this context, the distinct cellular pools of Axin may reflect the existence of different, active and inactive, complexes. The challenge will be to characterize the nature of these complexes and their regulation by upstream components of the pathway.

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