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

François Fagotto,* Eek-hoon Jho,† Li Zeng,‡ Thomas Kurth,* Thomas Joo,* 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 functions 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 Dishevelled (Dsh) revealed strong colocalization with Axin, suggesting that Dsh can interact with the Axin/APC complex and may thus modulate its activity.

Key words: β-catenin • glycogen synthase kinase 3β (GSK3β) • adenomatous polyposis coli (APC) • Dishevelled (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 (Gluecksohn-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 Dishevelled [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 through components of the Wnt pathway and suggested

1. Abbreviations used in this paper: aa, amino acid; APC, adenomatous polyposis coli; coIP, coimmunoprecipitate/coimmunoprecipitation; DAP1, 4,6-diamidino-2-phenylindole; Dsh, Dishevelled; FL, full-length; GSK3β, glycogen synthase kinase 3β; HA, hemagglutinin; HA–Dsh, hemagglutinin-tagged Dishevelled; IF, immunofluorescence; IP, immunoprecipitation; Myc–Axin, Myc-tagged Axin; PAAb, polyclonal antibody; PP2A, protein phosphatase 2A; RGS, regulators of G-protein signaling; VSV–G vesicular stomatitis virus glycoprotein; VSV–APC, VSV–G-epitope-tagged APC.
that Axin functioned downstream of glycogen synthase kinase 3β (GSK3β) and upstream of β-catenin (Zeng et al., 1997).

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; Miller 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. Cytosolic β-catenin levels are very low because free β-catenin is a target for GSK3β-dependent phosphorylation and is degraded rapidly via the ubiquitin pathway. In the presence of a Wnt signal, GSK3β 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 GSK3β activity or the accessibility of β-catenin to GSK3β. 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 (M uenemitsu 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 (R ocheleau et al., 1997; V leminckx 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 GSK3β 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 Axin in its ability to ventralize frog embryos (an in vivo 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 (Wnt, Frizzled, Dsh) that affect GSK3β activity or the accessibility of β-catenin to GSK3β.

Materials and Methods

Plasmid Construction

To express Myc-tagged forms of Axin, the coding region of mouse Axin (form 1) sequence was inserted downstream of the SP6 promoter in the vector pCS2-MT (Rupp et al., 1994). DNA inserts amplified by PCR with Pfu DNA polymerase (Stratagene) were used for the construction of plasmids containing small fragments, such as Ax455–552, Ax497–672, etc. Full-length (FL) X enopus APC cDNA (V leminckx et al., 1997) was inserted downstream of the CMV/SP6 promoter in the pCS2 vector, with a 5’ SV5–VSV-G (vesicular stomatitis virus glycoprotein) tag (Y TDIE MNR- LGK). Human Myc epitope-tagged APC constructs used for direct binding assays were described previously (V leminckx et al., 1997). PET32 vector (Novagen, Inc.) was used to produce the His S tagged Axin fusion vector (gift of Dr. U. Rothbächer, University of Marseille, France), HA-tagged X enopus β-catenin (F agotto et al., 1996), hemagglutinin (HA)-tagged X enopus β-catenin (F unayama et al., 1995), HA-tagged X enopus D sh (gift of Dr. U. Rothbächer, University of Marseille, France), HA-tagged dominant negative human GSK3β (gift of Dr. X. He, Harvard Medical School, Boston, MA).

Antibodies

A ntitoxins were purchased from the indicated sources: anti-β-catenin mouse mAb, clone 14, and anti-GSK3β mAb, clone 7, Transduction Laboratories; anti-VSV-G mAb P5D4, Boehringer Mannheim; anti-Myc 9E10.2 mAb, Calbiochem-Novabiochem; anti-HA–tag rabbit polyclonal antibodies (pAb), Santa Cruz Biotechnology; and anti-β-galactosidase rabbit pAb, Cappel Laboratories and O ragon Biof K Teka Korp. Anlti-HA mAb 12CA5 was a gift from Dr. P. M cCrea (M.D. Anderson Cancer Center, Houston, TX).

Tissue Culture and Transient Transfection

293 cells, obtained from the American Type Culture Collection, were cultured in DMEM/F12 medium (Mediatech) supplemented with 10% FBS.
Cell Fractionation

Early cleaving embryos were coinjected with 1 ng Myc-tagged A axin (Myc-A axin) and 3 ng β-galactosidase mRNA. 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 D TT, 1 mM EDTA supplemented with protease inhibitors. The homogenate was centrifuged for 5 min at 1,500 g, and the low speed supernatant was extracted in NP-40 buffer. The low speed supernatant was fractionated further by centrifugation for 30 min at 100,000 g in a tabletop ultracentrifuge (TL-100; Beckman Instruments Inc.) into a high speed pellet and supernatant. The fractions were analyzed for Myc- A axin and β-galactosidase by SDS-PAGE and immunoblot.

Con A Precipitation

FL A axin or various mutant constructs mRNA were injected into 4–8–stage cell embryos. A t stage 9–10, pools of six embryos were extracted in 500 μl NP-40 buffer and each extract was incubated with 50 μl of Con A –agarose beads (75% slurry; Sigma Chemical Co.) for 1–2 h at 4°C. The beads were spun down, the supernatant was collected (unbound fraction), the beads washed three times with 1 ml NP-40 buffer, and extracted by boiling in SDS-PAGE sample buffer (bound fraction). Levels of Myc-A axin constructs in bound and unbound fractions were analyzed by SDS-PAGE and immunoblot using the anti-Myc 9E10 antibody.

β-catenin Stability

HA-tagged β-catenin mRNA (75 pg) was coinjected with β-galactosidase mRNA (control) or various A axin mutant mRNA s. A mounts of mRNA injected were the following: β-galactosidase, 1 ng; FL A axin (A ax12-956), 1 ng; A ax12-956, 0.5 ng; A ax194-530, 0.5 ng; A a194-672, 0.25 ng; A ax531-931, 1 ng; A ax531-956, 0.5 ng; and A ax531-956, 0.5 ng. Total amounts of injected mRNA were adjusted to 1.075 ng by addition of β-galactosidase mRNA N. In some experiments (see Fig. 5 C), higher levels of β-catenin were tested using 0.75–1.5 ng mRNA. 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 axin mutant levels using the 9E10.2 mAb

Immunofluorescence (IF)

Stage 9–11 embryos were fixed in 4% paraformaldehyde, 100 mM H epes, pH 7.4, 100 mM NaCl for 1 h at room temperature, then in D en't s fixative (20% D M SO, 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). For double staining, sections from embryos coinjected with Myc-A axin and HA-tagged D sh (HA-D sh) mRNA s (1 ng each) were stained simultaneously with anti-Myc rabbit pAb and anti-HA mAb 12CA5, followed by A lex488 goat anti-rabbit and Cy3 donkey anti-mouse (Dianova) secondary antibodies.

For localization of Myc-A axin in cultured cells, HeLa cells cultured in DM EM were transfected with pC52-Myc-A axin using LipofectA mine (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 9E10.2 mAb and Cy3 goat anti-mouse (Dianova) secondary antibodies. Nuclei were counterstained with D A PI. Samples were observed with an A xipian epifluorescence microscope (Zeiss) using standard fluorescent and Cy3 filters, and digital images were collected using a camera (768x576 CCD color video; Sony).

Electro Microscopy and Immunogold Labeling

Preembedding labeling was performed as described (K urth, 1997; Fagotto, 1999). In brief, embryos expressing FL Myc-A axin or Myc-A a531-810 were fixed at stage 10 with 4% paraformaldehyde, 0.02% glutaraldehyde,
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 GSK3β 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 rAxin corresponding to aa 561–630 of mA xin 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 could also bind to the NH2-terminal region of APC (APC21), which contains the Armadillo repeats and 15 aa repeats (Fig. 2 C). However, we also found that a second region of Axin, between aa 96–253, could bind directly to the NH2-terminal region of APC (APC21), which contains the Armadillo repeats and 15 aa repeats (Fig. 2 C). colPs of the Axin mutants with VSV–A PC generally were dependent on the RGS domain; all mutants containing this domain were able to 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 x12-355 and A x12-167 could coIP with VSV–A PC, probably via β-catenin (Fig. 2 D). The second APC binding region (aa 96–253) was not sufficient for coIP with VSV–A PC (Fig. 2 D, A xA 251-351). Whether A x12-355 or A x12-167 could coIP with VSV–A PC could not be determined because expression of these mutant Axins 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 x12-353 caused a similar mobility shift, whereas A xA 323-351, A x12-355, and A x497-672, which lack either the GSK3β or A PC binding site, did not (Fig. 2 F). It has been shown that phosphorylation of APC by GSK3β (Rubinfeld et al., 1996) can be stimulated by Axin in vitro (Hart et al., 1998).
To test whether this Axin-induced mobility shift was due to phosphorylation, the immunoprecipitated proteins were treated with α-protein phosphatase before immunoblot analysis with anti-VSV. This treatment eliminated the mobility shift, indicating that it was due to phosphorylation (Fig. 2 F). This suggests that binding of A PC and G SK 3β to A xin promotes the phosphorylation of A PC in vivo, presumably by G SK 3β (Rubinfeld et al., 1996).

**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 Xenopus embryos, is due to its inhibitory effect on the Wnt signaling pathway (Zeng et al., 1997). Therefore, we used this assay to delimit the sequences in A xin required for its negative effects on signaling through the Wnt pathway. 22 mutant 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 dorsalized). The amount of injected mRNA s was systematically titrated to obtain comparable levels of expression 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 internal deletion of the R GS domain (A x1251-351) eliminated the ability to ventralize and instead caused dorsalization. Deletion of the G SK 3β and β-catenin binding sites (A x352-631) also abolished ventralizing activity. A small fragment containing the G SK 3β and β-catenin binding sites (A x497-672) was insufficient to ventralize the embryo, although similar A xin fragments were able to promote phosphorylation of β-catenin in vitro (Ikeda et al., 1998). A fragment containing only the R GS domain (A x194-353) was also ineffective.

Successive truncation from the NH2 terminus of A xin confirmed the importance of the R GS domain for ventralization. Whereas removal of the first 193 aa had no significant effect, further truncation to aa 331 eliminated ventralizing activity and resulted in dorsalizing activity, similar to the internal R GS deletion (Fig. 4, D and F). Truncation at aa 531, removing both the R GS domain and G SK 3β binding region, eliminated all activity (Fig. 4 C) as did truncation to aa 810 (A x810-956).

Mutant A xins with NH2-termini at aa 194 were subjected to COOH-terminal truncation to examine the importance of the D I X, P P A2 binding, β-catenin binding, and G SK 3β binding domains. Removal of the D I X domain (A x194-810) had little if any effect, whereas removal of the D I X and P P A2 binding domains (A x194-672) caused an increase in ventralizing activity (Fig. 4 B). This observation is consistent with the hypothesis that the binding of P P A2 to the A xin complex may negatively regulate the phosphorylation of β-catenin by G SK 3β (Hsu et al., 1999). Further truncation to aa 531, removing the β-catenin binding site, abolished ventralizing activity, and instead 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 NH2-terminal and the COOH-terminal regions (A x194-672 and A x194-810) also showed dorsalizing activity, as discussed below.

When the NH2 terminus of A xin was left intact, trunca-
tion of the COOH terminus to remove the D1X domain, or both the D1X 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 (Ax531-810).

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 caused 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 4 F, 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...
was observed only when high concentrations of RNA were injected. When the amount of injected RNA was titrated down to yield expression levels at which Ax x331-956 showed optimal activity, only Ax x331-956 was active (Table II).

**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 Xenopus 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. 2 B, FL Axin caused a dramatic decrease in exogenous β-catenin. Several mutant Axin constructs were coexpressed similarly; mutants with ventralizing activity (Ax12-531 and Ax194-672) also proved to be effective in reducing β-catenin levels. A mutant lacking ventralizing activity (Ax531-956) had no effect on β-catenin levels. On the other hand, mutants with strong dominant negative activity (Ax1251-351 and Ax331-956), induced a clear increase in β-catenin levels. Unexpectedly, mutant Ax194-531, 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 Ax x331-956 was also tested on the ventral side, where the β-catenin degradation machinery is maximally active. Under these conditions, stabilization of β-catenin by Ax x331-956 could be observed even in total extracts (Fig. 2 C). NH2 terminally deleted β-catenin (hemagglutinin epitope-tagged ΔNH2 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. 2 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 Xenopus embryos. A s 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

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

$^1$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.

$^2$Percent ventralization indicates the frequency of embryos with a DAI < 4.

$^3$DAI, dorsal anterior index; DAI 0, completely ventralized; DAI 5, normal; and DAI 10, completely dorsalized.

$^4$(∗), Dorsalized embryos obtained by dorsal injections cannot be scored accurately for DAI, because DAI values >5 are based on a somewhat different phenotype (LiCl-treated embryos). (d), some dorsalized embryos. (∗∗), dorsalized phenotype at high concentrations.

Number of experiments.

(Fig. 6, A and A’, arrows). The rest of the cytoplasm was devoid completely of the signal. In addition, some plasma membrane staining was also observed. However, the membrane staining was quite variable: absent in many cells, weak in others (Fig. 6, A and A’’, arrowhead), and very strong in a few rare cells (not shown). The punctate pattern and the absence of diffuse cytoplasmic staining were observed at all mRNA concentrations used from 0.15 ng, the limit of detection by IF, to 2 ng. A similar pattern was observed in A xin-transfected cultured H eLa (Fig. 6, B and B’’) and A 6 cells (not shown).

Myc-A xin localization in X enopus embryos was further studied at the EM level by two different techniques: on-section staining of Lowycryl sections (Fig. 7, A–C) and preembedding labeling using Nanogold and silver enhancement (Fig. 7, D–F). Notwithstanding differences in ultrastructure preservation and labeling sensitivity (see legends), both methods gave similar results. Consistent with IF data, Myc-A xin was found to be concentrated highly in discrete areas of the cell. These areas were characterized by clusters of vesicles (asterisks) surrounded by gold-decorated electron dense material (arrows). Labeled clusters varied largely in size and density, apparently as a function of expression levels. Part of a loose cluster is shown in Fig. 7 C. Small groups of gold particles associated with a few vesicles and electron dense material could be resolved, probably corresponding to the individual spots detected by IF (Figs. 6 A’ and 7 A, arrows). 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 xΔ531-810 that is consistent with IF results (Fig. 3).

Consistent with the IF data, A xin was found to be largely particulate/sedimentable in differential centrifugation experiments (Fig. 8 B). On the other hand, it was completely solubilized in the presence of a mild nonionic detergent, NP-40 (Fig. 8 C). Thus, the sedimentation properties of A xin are not due to interaction with detergent-insoluble cytoskeletal elements. In the presence of NP-40, A xin could be partially precipitated using Con A beads (Fig. 8 C), indicating that a pool of A xin is associated with a membrane glycoprotein. We believe that this A xin-membrane association involves the plasma membrane pool of A xin, but not that in the intracellular spots, be-
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 X. laevis is not known). Thus, we compared the localization of coexpressed Myc- and HA–Dsh by double IF. We observed a very good colocalization of these two proteins (Fig. 6, C and G), and Ax12-355 (Fig. 9 J).

For instance, Ax12-531 binds very efficiently to Con A, and the COOH-terminal 146-aa resulted in localization mainly at the membrane, with little or no labeling of the spots, e.g., Ax531-630 (found mostly in spots) presented on January 22, 2018 jcb.rupress.org Downloaded from

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

<table>
<thead>
<tr>
<th>Mutant</th>
<th>Amount mRNA injected</th>
<th>Total injected embryos</th>
<th>Percent DA</th>
<th>Dorsalization</th>
<th>cDA</th>
<th>pDA</th>
<th>vDA</th>
<th>N</th>
<th>No. exp</th>
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<tr>
<td>FL (12-956)</td>
<td>1–4</td>
<td>58</td>
<td>0</td>
<td>-</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>58</td>
<td>4</td>
</tr>
<tr>
<td>Δ251-351</td>
<td>1–2</td>
<td>148</td>
<td>80</td>
<td>+++</td>
<td>54</td>
<td>65</td>
<td>12</td>
<td>17</td>
<td>9</td>
</tr>
<tr>
<td>Δ352-631</td>
<td>1</td>
<td>39</td>
<td>0</td>
<td>-</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>39</td>
<td>3</td>
</tr>
<tr>
<td>331-956</td>
<td>0.25</td>
<td>59</td>
<td>19</td>
<td>+</td>
<td>0</td>
<td>11</td>
<td>0</td>
<td>11</td>
<td>3</td>
</tr>
<tr>
<td>331-956</td>
<td>1</td>
<td>129</td>
<td>87</td>
<td>+++</td>
<td>83</td>
<td>29</td>
<td>3</td>
<td>14</td>
<td>4</td>
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<tr>
<td>531-956</td>
<td>0.5–1.5</td>
<td>62</td>
<td>0</td>
<td>-</td>
<td>0</td>
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<td>0</td>
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<tr>
<td>194-531</td>
<td>0.5</td>
<td>97</td>
<td>24</td>
<td>+</td>
<td>9</td>
<td>14</td>
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<td>194-353</td>
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<tr>
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<tr>
<td>531-810</td>
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<td>-</td>
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<td>0</td>
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<td>2</td>
</tr>
<tr>
<td>12-810Δ251-351</td>
<td>0.25</td>
<td>23</td>
<td>0</td>
<td>-</td>
<td>0</td>
<td>0</td>
<td>2</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>12-810Δ251-351</td>
<td>0.5–2†</td>
<td>149</td>
<td>56</td>
<td>+</td>
<td>15</td>
<td>69</td>
<td>6</td>
<td>59</td>
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<td>7</td>
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<tr>
<td>12-531Δ251-351</td>
<td>0.25*</td>
<td>28</td>
<td>0</td>
<td>-</td>
<td>0</td>
<td>0</td>
<td>0</td>
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</tr>
<tr>
<td>12-531Δ251-351</td>
<td>0.5–2†</td>
<td>53</td>
<td>30</td>
<td>+</td>
<td>4</td>
<td>12</td>
<td>6</td>
<td>31</td>
<td>4</td>
</tr>
<tr>
<td>497-672</td>
<td>0.5</td>
<td>45</td>
<td>0</td>
<td>-</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>2</td>
</tr>
<tr>
<td>403-600</td>
<td>0.5–1</td>
<td>91</td>
<td>3</td>
<td>-</td>
<td>0</td>
<td>3</td>
<td>0</td>
<td>88</td>
<td>4</td>
</tr>
</tbody>
</table>

* Expression levels equivalent to 1–2 ng FL Axin mRNA.
† Expression levels higher than for FL and 12-956Δ251-351 Axin.
‡ Expression levels equivalent to 1–2 ng FL Axin mRNA.

DA, duplicated axis; percent DA, all duplicated axis (cDA = total injected embryos; cDA, complete DA; pDA, partial DA; vDA, vestigial DA; N, normal embryos; and No. Exp, number of experiments.)

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 localize primarily in the spots and less at the plasma membrane (Fig. 3 and Fig. 9, B and C). Deletion of the GSK 3b and β-catenin binding sites (A x1Δ352-631) 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., A x12-810 (not shown) A x12-672 (Fig. 9 F), A x12-531 (Fig. 9 G), and A x12-355 (Fig. 9 J).

Forms of Axin lacking the NH2-terminal half displayed a mostly diffuse cytoplasmic localization, e.g., A x497-672 (Fig. 9 D). When the APC and GSK 3b-binding domains were left intact, there seemed to be some enrichment at the cell periphery (Fig. 9 E, A x194-956), 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 GSK 3b-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, A x12-531 binds very efficiently to Con A, whereas A x531-956 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 A xΔ351-630 (found mostly in spots) bind to Con A (Fig. 8 C and data not shown).
Figure 5. Expression of Axin and Axin mutants affects β-catenin stability. (A) Diagram of Axin molecule. (B) Dorsal injection of FL Axin and several mutant constructs downregulate free β-catenin levels. HA-tagged β-catenin mRNA (75 pg) was coinjected with β-galactosidase mRNA (control), or the indicated Myc-tagged forms of Axin, in the dorsal side of early cleaving embryos. mRNA amounts (see Materials and Methods) were selected to produce equivalent levels of expression of Axin constructs, and β-galactosidase mRNA was added to equalize amounts of injected mRNA. Extracts from late blastula embryos were depleted of cadherin-bound β-catenin using Con A beads and the levels of free HA-β-catenin were analyzed by immunoblot. Duplicate samples correspond to two independent pools of seven embryos each. FL and several mutant forms (Ax12-531, Ax194-531, and Ax194-672) strongly reduced β-catenin levels. On the other hand, Ax1251-351 and Ax331-956 caused an increase in β-catenin levels. Ax194-531 had no effect. (C) Stabilization of total β-catenin by ventral injection of Ax1251-351. HAsβ-catenin mRNA was coinjected with β-galactosidase, Ax1251-351, FL Axin, or HA-dnGSKβ-catenin mRNA in the ventral side of cleaving embryos. Total levels of HAsβ-catenin were compared directly by immunoblot. Ax1251-351, but not FL Axin, induced an increase in β-catenin comparable to the increase obtained with dnGSKβ-catenin (Yost et al., 1996). The lower panels show expression of HAsβ-catenin and of the Myc-tagged Axin constructs. (D) Axin downregulates FL β-catenin, but not NH2-terminally truncated β-catenin. HA-tagged β-catenin or ΔN β-catenin mRNA (750 pg) were coinjected dorsally with β-galactosidase mRNA (control, 2 ng) or FL Axin mRNA (2 ng). Total levels of HA-β-catenin were compared.

Discussion

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 GSK3β 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 GSK3β and its subsequent degradation (Hart et al., 1998; Ikeda et al., 1998; Toh 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, GSK3β, 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, GSK3β, 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 GSK3β 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 GSK3β with Axin 12-526, we detected weak interaction of GSK3β 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 repeats. 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 GSK3β (Ikeda et al., 1998) and the ability of Axin mutants to coIP with GSK3β (Fig. 4). Axin mutants lacking this region, some of which were able to coIP with APC and/or β-catenin, all failed to coIP with GSK3β, suggesting that GSK3β 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 Ax331-956, which lacks any direct binding site for APC. Similarly, β-catenin could coIP not only with all Axin mutants containing aa 600-622, but also with Ax1251-351 and Ax194-531. Both of these discrepancies likely are due to the ability of APC and β-catenin to bind to 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 GSK3β 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 (Ax194-353), while able to coIP with APC, did not coIP...
with β-catenin. Conversely, A x531-956 was found to colocalize 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, suggesting that GSK3 β is responsible for this modification. It previously 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 (Rubinfeld et al., 1996). Our observations argue that A xin performs 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 ability of A xin mutants to ventralize frog embryos and to lower the levels of coinjected HA-tagged β-catenin, presumably 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-catenin 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 colocalize with β-catenin in 293 cells, apparently via an indirect interaction. Why the NH2-terminal sequence can substitute for the β-catenin binding site is not clear, but this could be related to its ability to bind to A PC or its influence on the intracellular localization of A xin.

Fragments of A xin containing the GSK3 β and β-catenin binding sites, but lacking the RGS domain, can promote the phosphorylation of β-catenin by GSK3 β in vitro (Ikeda et al., 1998) as well as the degradation of endogenous β-catenin in cultured SW480 cells (Hart et al., 1998). However, in our experiments, all forms of A xin lacking the RGS domain either had no effect on axis formation and β-catenin levels or they dorsalized rather than ventralized the frog embryo and raised β-catenin levels. These discrepancies may be due to the peculiar properties of SW480 cells that lack FL A PC and have high levels of soluble β-catenin. In contrast, we used very low levels of exogenous β-catenin that can still be effectively regulated by the Wnt pathway (Yost et al., 1996). In other ex-
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 (2x) 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 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.

Figure 7. 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.

Experiments (Fig. 5 C), we found that FL Axin was able to downregulate even very large amounts of coinjected 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 A PC. Consistent with this observation, the Axin-like protein Conductin/A xil/Axin-2 appears to behave similarly. Indeed, a ΔR GS 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 A PC (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 A PC-dependent, and one A PC-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 A PC (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, ΔR GS 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 A PC, 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 A PC may interfere with Axin activity.

In embryos, deletion of the RGS domain caused a strong dominant negative effect. The self-binding region (including the D1X 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-
nullous Axin. However, forms of Axin lacking the DIX domain also showed weak or moderate dorsalizing activity (Ax12-810-251-351 and Ax12-531-251-351), 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 bind to GSK3β but not to APC, thus, interfering with the formation of the complete complex. Aiso, two mutants lacking both the NH2 and COOH termini (Ax194-672 and Ax194-353) efficiently ventralized at low concentrations, but dorsalized at high concentrations. This dual activity might be related to their subcellular localization (see below).

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 (Su 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. (2) 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 unconluded 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 A x12-531. 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 Ax194-672 and Ax194-810 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 $\beta$-catenin: When $\beta$-catenin levels are low, $\beta$-catenin degradation would depend primarily of an Axin•$\beta$-catenin•APC complex, whereas when $\beta$-catenin levels are high, $\beta$-catenin•Axin complexes may form and function in the absence of APC. Both mechanisms may be physiologically important: in the absence of Wnt signal, very low levels of free $\beta$-catenin might be maintained by the combined action of Axin and APC. However, after a burst of Wnt signal, excess $\beta$-catenin would be first downregulated by an APC-independent cause, before fine tuning by 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 $\beta$-catenin stabilization by inhibiting GSK3\beta activity (for review see Miller and Moon, 1996). However, GSK3\beta inhibition could hardly account for the specificity of the pathway, considering the many other substrates and pleiotropic functions of GSK3\beta. We now know that binding of GSK3\beta to Axin is required for phosphorylation of Axin, APC, and $\beta$-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-GSK3\beta 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.

The authors thank Anne Schohl for technical assistance.

This work was supported by a National Institutes of Health grant GM 56934 to F. Costantini.

Received for publication 28 December 1998 and in revised form 24 March 1999.

References


Yamamoto, H., S. Kishiha, T. Uchi, S. Ikena, S. Koyama, M. Ahashima, and A. Kikuchi. 1998. A xil, a member of the A Xin family, interacts with both glyco-


Yanagawa, S., F. van Leeuwen, A. Wodarz, J. Klangsmit, and R. Nuise. 1995. The dishevelled protein is modified by wingless signaling in Dro-


Biol. 6:1302–1306.

