Inhibition of Wnt Signaling Pathway by a Novel Axin-binding Protein*

Received for publication, July 7, 2000, and in revised form, August 14, 2000
Published, JBC Papers in Press, August 15, 2000, DOI 10.1074/jbc.M005984200

Takayuki Kadoya‡, Shosei Kishida‡, Akimasa Fukui, Takao Hinoi‡, Tatsuo Michiue**, Makoto Asashima***, and Akira Kikuchi‡‡

From the Department of Biochemistry and **Second Department of Surgery, Hiroshima University School of Medicine, 1-2-3 Kasumi, Minami-ku, Hiroshima 734-8551, ¶PRESTO, Japan Science and Technology Corporation, Hiroshima 734-8551, the Department of Life Science (Biology), and **CREST Project, University of Tokyo, 3-8-1, Komaba, Meguro-ku, Tokyo 153-8902, Japan

Axin forms a complex with adenomatous polyposis coli gene product, glycogen synthase kinase-3β (GSK-3β), β-catenin, Dvl, and protein phosphatase 2A and functions as a scaffold protein in the Wnt signaling pathway. In the Axin complex, GSK-3β efficiently phosphorylates β-catenin, which is then ubiquitinated and degraded by proteasome. We isolated a novel protein that binds to Axin and named it Axam (for Axin associating molecule). Axam formed a complex with Axin in intact cells and bound directly to Axin. Axam inhibited the complex formation of Dvl with Axin and the activity of Dvl to suppress GSK-3β-dependent phosphorylation of Axin. Furthermore, Axam induced the degradation of β-catenin in SW480 cells and inhibited Wnt-dependent axis duplication in Xenopus embryos. These results suggest that Axam regulates the Wnt signaling pathway negatively by inhibiting the binding of Dvl to Axin.

Wnt proteins constitute a large family of cysteine-rich secreted ligands that control development in organisms ranging from nematode worms to mammals (1, 2). At the cellular level, Wnt regulates proliferation, morphology, motility, and fate. The outlines of the Wnt signal transduction pathway were first elucidated by a genetic analysis of Wingless pathway during the development of segmental polarity in Drosophila and extended through studies of embryonic axis formation in Xenopus. A number of components of the Wingless pathway have been identified (3–5). In the current model, the serine/threonine kinase Zw3/shaggy targets cytoplasmic Armadillo protein for degradation in the absence of Wingless. As a result, cytoplasmic Armadillo levels are low. When Wingless interacts with its cell-surface receptor, D-Frizzled2, the signal antagonizes the action of Zw3 through Dishevelled. Armadillo is no longer degraded, resulting in its accumulation in both cytoplasm and nucleus, where it binds to Pangolin, a transcription factor, and stimulates gene expression. The intracellular components of the Wingless pathway are shared by multicellular animals. In Xenopus and mammals, the Frizzled homolog frizzled, the Dishevelled homolog Dvl, the Zw3 homolog GSK-3β, the Armadillo homolog β-catenin, and the Pangolin homolog T cell factor/lymphocyte enhancer binding factor family are involved in the transduction of the vertebrate Wnt signaling.

Thus, the mechanism of the Wnt signaling pathway has been proposed based on Drosophila genetic evidence, but the biochemical relationship between the components in the pathway has not long been clarified. Discovery and functional analyses of Axin have provided new clues to understand how the Wnt signaling pathway is regulated (6, 7). Axin was originally identified as a product of the mouse Fused locus (6). The mouse mutant Fused is recessive-lethal; mutants have a duplication of the embryonic axis (8, 9). Expression of Axin in Xenopus embryos causes strong axis defects, and co-expression of Axin inhibits the Wnt-dependent axis duplication (6). Thus, Axin is a negative regulator of the Wnt signaling pathway and inhibits axis formation. We have identified rat Axin (rAxin) and its homolog, Axil (for Axin like), as GSK-3β-interacting proteins (7, 10, 11). Conductin has been identified as a β-catenin-binding protein (12) and is identical with Axil. Both Axin and Axil bind not only to GSK-3β but also to β-catenin and APC (10–16) and promote GSK-3β-dependent phosphorylation of β-catenin and APC (10, 11, 15, 17). Phosphorylated β-catenin forms a complex with Fbw1 (βTrCP/FWD1), a member of the F box protein family, resulting in the degradation of β-catenin by a ubiquitin-proteasome pathway (18–20). The phosphorylation of APC enhances its binding to β-catenin (21). Axin is also phosphorylated by GSK-3β and stabilized by its phosphorylation in contrast to β-catenin (22), and the phosphorylation increases the binding of Axin to β-catenin (23, 24). Therefore, the phosphorylation of β-catenin, APC, and Axin leads to the degradation of β-catenin. Dvl interacts with Axin (25–27), and this interaction inhibits GSK-3β-dependent phosphorylation of β-catenin, APC, and Axin in the Axin complex (22, 26). It has been shown that Dvl displaces GSK-3 from its complex with Axin-related protein in Xenopus (28). Furthermore, Wnt-3a-de-
dependent accumulation of β-catenin and down-regulation of Axin are suppressed by expression of a dominant negative form of Dvl-1 (22). These results suggest that the signal of Wnt to Axin is transmitted through Dvl. PP2A and PP2C bind to Axin and Dvl, respectively (17, 29, 30), and they dephosphorylate APC and Axin (17, 30). Thus, Axin may be a scaffold protein, in that it binds to several signaling molecules to create a multiprotein complex.

To find additional roles of Axin in the Wnt signaling pathway, we tried to identify a new protein that binds to Axin. We show here a novel Axin-binding protein that we designated Axam (for Axin associating molecule). Axam binds directly to Axin and competes with Dvl for its binding to Axin, thereby inhibiting the activity of Dvl-1 to suppress the phosphorylation of Axin by GSK-3β. Furthermore, Axam degrades β-catenin in SW480 cells, human colon cancer cells, and inhibits axis formation in Xenopus embryos. These results suggest that Axam regulates the Wnt signaling pathway negatively by inhibiting the binding of Dvl to Axin.

EXPERIMENTAL PROCEDURES

Materials and Chemicals—The cDNAs of human Dvl-1 and Xenopus β-catenin (Xβ-catenin) were provided by B. Dallapiccola and G. Novelli (Vergata University, Rome, Italy) and D. Kimelman (University of Washington, Seattle), respectively. Recombinant baculovirus expressing GST-Dvl-1 (full length) was provided by Y. Matsuura (National Institute of Infectious Diseases, Tokyo, Japan). The anti-GST and anti-MBP antibodies were supplied by M. Nakata (Sumitomo Electronics, Yokohama, Japan). MBP and GST fusion proteins except for GST-Dvl-1 (full length) were purified from Escherichia coli according to the manufacturer’s instructions. The anti-Myc antibody was prepared from 9E10 cells. The anti-GSK-3β, anti-β-catenin, anti-PP2A antibodies, and the anti-GFP antibody were purchased from Transduction Laboratories (Lexington, KY) and Molecular Probes, Inc. (Eugene, OR), respectively. [α-32P]dCTP was obtained from Amersham Pharmacia Biotech. LipofectAMINE 2000 (Life Technologies, Inc.) and TransFast™ (Promega Corp., Madison, WI) were used for transfection of COS and SW480 cells, respectively. Other materials were from commercial sources.

Plasmid Construction and Transfection—pBTM116HA/rAxin, pMAL-c2/rAxin, pMAL-c2/rAxin-(298–506), pBJ-Myc/rAxin, pEF-BOS/Myc-Axin (1–713), pEF-BOS/Myc-rAxin (508–832), pBJ-Myc/rAxin-(713–832), pBSKS/Axam-(508–832), pMALc-2/rAxin-(713–832), pMAL-c2/rAxin-(508–832), pGEX-2T/rAxin-(1–229), pMAL-c2/rAxin-(1–229), pMAL-c2/rAxin-(508–832), pEGFP-C2/Axam-(72–400), pEGFP-C3/Axam-(381–521), and pEGFP-C3/Axam-(521–588). In these plasmids, some plasmid constructions were done by digesting the original plasmids with restriction enzymes and inserting the fragments into the vectors. The other constructions were done by inserting the fragments generated by Expand™ High Fidelity PCR.
system (Roche Molecular Biochemicals) into the vectors. The entire polymerase chain reaction products were sequenced, and the structures of all plasmids were confirmed by restriction analysis.

A Novel Axin-binding Protein

Detection of Endogenous Axam by the Anti-Axam antibody—The anti-Axam rabbit polyclonal antibody was generated by immunizing rabbits with Axam-(72–588). Seven-week-old male rats of the Wistar strain were sedated with CO2 gas and sacrificed by decapitation. Cerebrum and cerebellum were immediately excised and homogenized in 9 ml/g of tissue (wet weight) of homogenizing buffer (20 mM Tris/HCl, pH 7.5, 0.25 M sucrose, and 1 mM PMSF). 293, COS, L, F9, PC12, SW480, DLD-1, and RCN-9 cells (10-cm diameter dish) were lysed in 500 μl of the lysis buffer (20 mM Tris/HCl, pH 7.5, 150 mM NaCl, 10% glycerol, 1% Nonidet P-40, 20 μg/ml leupeptin, 20 μg/ml aprotinin, and 1 mM PMSF) and the homogenate was centrifuged at 15,000 × g for 10 min at 4 °C. These extracts of rat tissues (100 µg/lane) and various cell lines (60 µg/lane) were probed with the anti-Axam antibody.

Complex Formation of Axam with Axin in Intact Cells—To determine whether Axam interacts with Axin in intact cells, COS cells (6-cm diameter dish) transfected with pEGFP, pCGN-, pEF-BOS, and/or pBJ-derived plasmids were lysed in 200 μl of the lysis buffer. The supernatant (150 μg of protein) was immunoprecipitated with the anti-GFP or anti-Myc antibody and then the immunoprecipitates were probed with the anti-GFP, anti-Myc, anti-HA, anti-PP2A, anti-GSK-3β, and/or anti-β-catenin antibodies.

Immunofluorescence Study—SW480 cells expressing Myc-rAxin, GFP, GFP-Axam, or GFP-Axam-(72–588) grown on coverslips were fixed for 20 min in PBS containing 4% paraformaldehyde. The cells were washed with PBS three times and then permeabilized with PBS containing 0.2% Triton X-100 and 2 mg/ml BSA for 2 h. They were washed and incubated for 1 h with the anti-Myc or β-catenin and GFP antibodies. After being washed with PBS, the cells were further incubated for 1 h with Alexa 488-labeled goat anti-rabbit IgG and Alexa-594-labeled goat anti-mouse IgG. The coverslips were washed with PBS, mounted on glass slides, and viewed with a confocal laser-scanning microscope (TCS-NT®, Leica-laser-technik GmbH, Heidelberg, Germany). For multichannel imaging, each fluorescence was imaged sequentially to eliminate cross-talk between the channels.

Direct Interaction of Axam with Axin—To examine the interaction of Axam with Axin using the purified proteins in vitro, 1 μM GST-Axam and its deletion mutants were incubated with MBP-rAxin or its deletion mutants (40 pmol) immobilized on amylose resin in 40 μl of reaction mixture (50 mM Tris/HCl, pH 8.0, and 1 mM dithiothreitol) for 1 h at 4 °C. MBP-rAxin was precipitated by centrifugation, then the precipitates were probed with the anti-GST antibody.

Phosphorylation of Axin in Intact Cells—To observe the phosphorylation states of Axin in intact cells, COS cells expressing Myc-rAxin were treated with or without 100 nM okadaic acid for 12 h. The lysates were applied to a 6% SDS-PAGE gel and subjected to Western blot analysis with the anti-Axym antibody. When necessary, Myc-rAxin was immunoprecipitated with the anti-Myc antibody, and the immunoprecipitates were incubated with 6 units of alkaline phosphatase in 20 μl of reaction mixture (50 mM Tris/HCl, pH 8.0, and 1 mM dithiothreitol) for 1 h at 4 °C. MBP-rAxin was precipitated by centrifugation, then the precipitates were probed with the anti-Myc antibody.

Down-regulation of β-Catenin by Axam—SW480 cells (3.5-cm diameter dish) transfected with pEGFP-C2 or pEGFP-C2/Axam-(72–588) were washed with ice-cold PBS and suspended in 250 μl of the sonica-
A Novel Axin-binding Protein

**RESULTS**

Isolation of Axin-binding Protein—To identify proteins that physically interact with Axin, we conducted a rat brain cDNA library screening by the yeast two-hybrid method. From 1 × 10⁶ initial transformants, 2 clones were found to confer both the His⁺ and LacZ⁺ phenotypes on L40 expressing rAxin. Of these, a 1.6-kb cDNA insert was found to encode a sequence containing a long open reading frame and the consensus sequence for a stop codon. A full-length cDNA of this Axin-interacting protein was isolated from a rat brain cDNA library. This clone spanned a distance of 3.9 kb and contained an uninterrupted open reading frame of 1,764 base pairs, encoding a predicted protein of 588 amino acids with a calculated Mᵋ of 67,251 (Fig. 1A). The first ATG was preceded by stop codons in all three reading frames, and the 5’-noncoding region had a high percentage of GC base pairs (74%). The neighboring sequence of the first ATG was consistent with the translation initiation start proposed by Kozak (35). By searching several data bases, we found one human cDNA (AB037752) registered in GenBank™ and two mouse ESTs (BE289601 and BE310212) as highly conserved homologs of this Axin-interacting protein. As the amino acid sequence predicted from the human cDNA clone shares 88% amino acid identity with two-thirds of the C-terminal region of the rat Axin-binding protein, it may be a human counterpart. However, we did not find similar sequences in EST clones from *Xenopus, Drosophila,* or *Caenorhabditis elegans.* Since this Axin-interacting protein is a novel protein, we named it Axam. The region of amino acid 31–49 of Axam contains a PEST sequence. A single band of 3.9-kb Axam mRNA was ubiquitously detected in rat tissues as far as we examined, and it was highly expressed in cerebrum, lung, and testis (Fig. 1B). The anti-Axam antibody detected a protein with a molecular mass of 67 kDa in rat cerebrum and cerebellum. This protein was not observed when the antibody preincubated with MBP-Axam-(72–588) was used (data not shown). Its molecular mass is the same as that predicted from the amino acid sequence of Axam, suggesting that it is endogenous Axam. Furthermore, Axam was found in several cell lines including 293 (human kidney epithelial cell), COS (monkey kidney cell), L (mouse fibroblast), F9 (mouse teratocarcinoma cell), PC12 (rat pheochromocytoma cell), SW480 (human colon cancer cell), DLD-1 (human colon cancer cell), and RCN-9 (rat colon cancer cell) cells (Fig. 1C). Various mutants of Axin and Axam used in this study are shown in Fig. 1D.

Complex Formation of Axam and Axin in Intact Cells—To examine whether Axam forms a complex with Axin in intact cells, rAxin was co-expressed with Axam or its deletion mutants in COS cells (Fig. 2A, lanes 1–12). rAxin and Axam were tagged with Myc and GFP epitopes at their N termini, respectively. When the lysates co-expressing Myc-rAxin with GFP-Axam-(1–113) was immunoprecipitated with the anti-Myc antibody, GFP-Axam was detected in the Myc-rAxin immune complex (Fig. 2A, lane 19). Similarly, when the same lysates were immunoprecipitated with the anti-GFP antibody, Myc-rAxin was detected in the GFP-Axam immune complex (data not shown). Neither Myc-rAxin nor GFP-Axam was immunoprecipitated from the lysates expressing both proteins with non-immune immunoglobulin (data not shown). Axam-(72–588) is an original protein isolated as a binding partner of rAxin by yeast two-hybrid screening. GFP-Axam-(72–588) but not GFP-Axam-(1–113) was immunoprecipitated with Myc-rAxin (Fig. 2A, lanes 20 and 21). In residues 72–588, GFP-Axam-(72–400) but not GFP-Axam-(381–521) or GFP-Axam-(521–588) formed a complex with Myc-rAxin (Fig. 2A, lanes 22–24).

**FIG. 4.** Direct interaction of Axam with Axin. *A,* binding region of Axam for rAxin. Purified proteins (0.5 μg of protein each) used in this experiment were subjected to SDS-PAGE followed by Coomassie Brilliant Blue staining (lanes 1–5). The arrowheads indicate the purified proteins. After GST-Axam and its deletion mutants had been incubated with MBP-rAxin (full length) (lanes 6–8) or MBP (lanes 9–11) immobilized on amyllose resin, MBP-rAxin and MBP were precipitated by centrifugation. The precipitates were probed with the anti-GST antibody. *Input,* the Coomassie Brilliant Blue staining of MBP-rAxin or MBP used in each assay. *B,* dose dependence. The indicated concentrations of GST-Axam-(72–588) were incubated with MBP-rAxin immobilized on amyllose resin for 1 h. *C,* binding region of rAxin for Axam. After GST-Axam-(72–588) had been incubated with MBP-rAxin and its deletion mutants immobilized on amyllose resin, MBP fusion proteins were precipitated by centrifugation, and the precipitates were probed with the anti-GST antibody (upper panel) and stained with Coomassie Brilliant Blue (lower panel). The arrowheads indicate MBP-rAxin mutants used in this assay. The results shown are representative of four independent experiments.
To determine which region of rAxin forms a complex with Axin in intact cells, various deletion mutants of rAxin were expressed with Axin in COS cells (Fig. 2B, lanes 1–5). When the lysates expressing Myc-rAxin mutants were immunoprecipitated with the anti-Myc antibody, GFP-Axam-(72–588) was co-precipitated with Myc-rAxin-(full length) and Myc-rAxin-(1–437), Myc-rAxin-(298–506), or Myc-rAxin-(713–832), but not with Myc-rAxin-(1–229) or Myc-rAxin-(713–832) (Fig. 2B, lanes 6–10). These results suggest that Axam interacts with Axin in intact cells and that the region containing residues 114–588 of Axam and the region containing residues 507–712 of rAxin are mainly responsible for their interaction.

We next examined whether Axam and Axin co-localize in intact cells. When GFP-Axam-(72–588) was expressed alone in SW480 cells, it was present in the cytoplasm diffusely (Fig. 3A). Consistent with previous observations (25–27, 36), Myc-rAxin was observed (Fig. 3A, lanes 1–3, 5–7). SW480 cells, it was present in the cytoplasm diffusely (Fig. 3A). Consistent with previous observations (25–27, 36), Myc-rAxin was observed (Fig. 3A, lanes 1–3, 5–7). Since Dvl-1 also binds directly to this region of Axin (26), we examined whether Dvl and Axam share the same binding site on Axin. To this end, MBP-rAxin-(508–832) and GST-Dvl-1 were purified (Fig. 5A, lanes 1 and 2). GST-Axam-(72–588) and GST-Dvl-1 interacted with MBP-rAxin-(508–832) strongly and MBP-rAxin-(298–506) weakly but not to MBP-rAxin-(1–229) or MBP-rAxin-(713–832) (Fig. 4C). These results suggest that the region containing residues 508–712 of rAxin binds directly to that containing residues 114–588 of Axam, consistent with the results obtained with intact cells.

Inhibition of the Binding of Dvl to Axin by Axam—As shown in Figs. 2 and 4, Axam bound to residues 508–712 of Axin. Since Dvl-1 also binds directly to this region of Axin (26), we examined whether Dvl and Axam share the same binding site on Axin. To this end, MBP-rAxin-(508–832) and MBP-rAxin-(620–712) were purified (Fig. 5A, lanes 1 and 2). GST-Axam-(72–588) and GST-Dvl-1 interacted with MBP-rAxin-(508–832) (Fig. 5A, lanes 5 and 6) but not with MBP-rAxin-(620–712) (Fig. 5A, lanes 7 and 8). These results suggest that rAxin-(508–832) contains the Dvl- and Axam-binding sites. Therefore, we examined whether Axam inhibits the binding of Dvl to Axin in intact cells. Myc-rAxin, HA-Dvl-1, and GFP-Axam were co-expressed in COS cells (Fig. 5B, lanes 1–5, 11, and 12). When the expression levels of GFP-Axam increased, the amounts of MBP-(Fig. 5A, lanes 1–5) and MBP-(Fig. 5A, lanes 7–12) were reduced (Fig. 5A, lanes 9–11). Since GST-Axam (full length) is relatively hard to purify, GST-Axam-(72–588) was used for the biochemical studies described below. GST-Axam-(72–588) bound to MBP-rAxin in a dose-dependent manner (Fig. 4B). To examine which region of rAxin is required for its interaction with Axam, various deletion mutants including MBP-rAxin-(1–229), MBP-rAxin-(298–506), MBP-rAxin-(508–832), and MBP-rAxin-(713–832) were purified. GST-Axam-(72–588) bound to MBP-rAxin-(508–832) strongly and MBP-rAxin-(298–506) weakly but not to MBP-rAxin-(1–229) or MBP-rAxin-(713–832) (Fig. 4C). These results suggest that the region containing residues 508–712 of rAxin binds directly to that containing residues 114–588 of Axam, consistent with the results obtained with intact cells.
HA-Dvl-1 complexed with Myc-rAxin were reduced (Fig. 5B, lanes 6–10). Furthermore, expression of HA-Dvl-1 reduced the complex formation of Myc-rAxin with GFP-Axam (Fig. 5B, lanes 13 and 14). These results suggest that Axam and Dvl compete with each other for the binding to Axin in intact cells. However, Axam did not inhibit the complex formation of β-catenin, GSK-3β, or PP2A with Axin (Fig. 5C).

Effects of Axam on Phosphorylation of Axin—Dvl-1 inhibits the GSK-3β-dependent phosphorylation of Axin, β-catenin, and APC in the Axin complex in vitro (22, 26). We examined whether Axam affects this function of Dvl-1 in intact cells. When Myc-rAxin immunoprecipitated from COS cells with the anti-Myc antibody was applied to a 6% SDS-PAGE gel, a ladder of slower migrating bands of Myc-rAxin was observed (Fig. 6A, lane 1). These high molecular weight bands were decreased by the treatment with alkaline phosphatase (Fig. 6A, lane 2). When COS cells expressing Myc-rAxin were treated with okadaic acid, a protein phosphatase inhibitor, the high molecular weight band of Myc-rAxin was increased (Fig. 6A, lane 3). These results indicate that the slower migrating bands of Myc-rAxin are phosphorylated forms. By co-expression with HA-Dvl-1, Myc-rAxin migrated faster, suggesting that Dvl-1 inhibits the phosphorylation of Axin in intact cells (Fig. 6B, lanes 1 and 2). Additional expression of GFP-Axam reversed the mobility change of Myc-rAxin by HA-Dvl-1 (Fig. 6B, lane 3). Furthermore, when Myc-rAxin and GFP-Axam were co-expressed, Myc-rAxin exhibited a slower migration (Fig. 6B, lane 4). These results suggest that Axam inhibits the activity of Dvl-1 to suppress GSK-3β-dependent phosphorylation in the Axin complex, it may allow Axin to degrade β-catenin by inhibiting the binding of Dvl to Axin.

Effects of Axam on the Stability of β-Catenin—We examined the effects of Axam on the stability of β-catenin. Expression of GFP-Axam in SW480 cells, where cytoplasmic β-catenin is accumulated due to the truncation of APC, degraded β-catenin as did Axin (Fig. 7A, a and b). GFP-Axam-(72–588) but not GFP down-regulated β-catenin (Fig. 7A, c–f). These observations were confirmed by the Western blot analysis. Expression of GFP-Axam-(72–588) but not GFP reduced the protein level of β-catenin under the conditions that the levels of GSK-3β were not changed (Fig. 7B). These results suggest that Axam competes with Dvl for the binding to Axin, thereby releasing the negative regulation by Dvl for Axin, resulting in the down-regulation of β-catenin.

Effects of Axam on the Axis Formation in Xenopus Embryos—Finally, we used Xenopus embryos to examine the roles of Axam in the Wnt signaling pathway, because this signaling pathway is known to regulate the axis formation in Xenopus embryos (37). Dorsal injection of Axam-(72–588) inhibited the formation of axis in Xenopus embryos (Fig. 8A, a). The inhibitory effects on the formation by Axam was dose-dependent (Fig. 8B). Ventral injection of Axam had no effect on the formation of axis (Fig. 8, A, panel b, and B). Axam (full length) and Axam-(72–588) exhibited the same ability to inhibit the axis forma-
FIG. 8. Effects of Axam on the axis formation in Xenopus embryos. A, effects of Axam on the axis formation. Axam mRNA or Xglobin mRNA (400 pg) and the indicated amounts of other mRNAs were injected into the embryos. Panel a, dorsal injection of Axam; panel b, ventral injection of Axam. The following mRNAs were injected ventrally: Panel c, Xwnt-8 (50 pg) and Xglobin; panel d, Xwnt-8 (50 pg) and Axam; panel e, Dvl-1 (1 ng) and Xglobin; panel f, Dvl-1 (1 ng) and Axam; panel g, Xβ-catenin (1 ng) and Xglobin; panel h, Xβ-catenin (1 ng) and Axam; panel i, β-cateninSA (100 pg) and Xglobin; panel j, β-cateninSA (100 pg) and Axam. The arrows indicate the distinct branched axes that do not have head structures. B, average DAI of Xenopus embryos expressing Axam. The indicated mRNAs were injected into dorsal (lanes 1–5 and 7) or ventral (lane 6) blastomeres. Lane 1, Xglobin (1 ng); lane 2, Axam-(72–588) (50 pg); lane 3, Axam-(72–588) (100 pg); lane 4, Axam-(72–588) (200 pg); lanes 5 and 6, Axam-(72–588) (400 pg); lane 7, Axam (full length) (400 pg). D, dorsal injection; V, ventral injection. An average DAI is not an accurate concept but is used for illustrative purposes. C, frequency of Axis duplication. Lane 1, Xwnt-8 (50 pg) and Xglobin (400 pg); lane 2, Xwnt-8 and Axam (400 pg); lane 3, Dvl-1 (1 ng) and Xglobin; lane 4, Dvl-1 and Axam; lane 5, Xβ-catenin (1 ng) and Xglobin; lane 6, Xβ-catenin and Axam; lane 7, β-cateninSA (100 pg) and Xglobin; lane 8, β-cateninSA and Axam. Black bars indicate the complete axis duplication. It includes eyes and cement glands. White bars show the partial axis duplication characterized by no head structure but a distinct branched axis.

A Novel Axin-binding Protein

**DISCUSSION**

Axin acts as a negative regulator in the Wnt signaling pathway by interacting with several proteins including GSK-3β, β-catenin, APC, Dvl, and PP2A and stimulating the degradation of β-catenin (7). In this study, we have identified a new Axin-binding protein and named it Axam. Axam binds directly to region 508–620 of rAxin. Since Dvl also interacts directly with this region of Axin, Axam competes with Dvl for the binding to Axin. However, Axam does not affect the binding of GSK-3β, β-catenin, and PP2A to Axin. Dvl acts as a positive regulator of the Wnt signaling pathway since its expression induces the accumulation of β-catenin in fly and mammalian cells (26, 39) and induces the formation of a secondary axis in Xenopus embryos (37, 40). Together with the observations that Dvl antagonizes Axin in Xenopus embryos (6), our results suggest that Axam releases the inhibitory action of Dvl for Axin, thereby regulating the Wnt signaling pathway negatively.

We have already shown that Dvl inhibits the phosphorylation of β-catenin, Axin, and APC by GSK-3β in vitro (22, 26). There are three possible mechanisms by which Dvl inhibits the phosphorylation in the Axin complex. The first is that Dvl inhibits GSK-3β activity. This is supported by the observations that expression of Dvl reduces the phosphorylation of Tau which is another substrate of GSK-3β (41), that Wingless inhibits GSK-3β activity in mouse fibroblasts and Drosophila cells (42, 43), and that Dvl is located between Frizzled and GSK-3β genetically (1, 4). The second possibility is that Dvl activates protein phosphatases. This is supported by the findings that PP2C binds to the PDZ domain of Dvl and that expression of PP2C induces the dephosphorylation of Axin (30). PP2A, another type of protein phosphatase, also interacts with Axin and inhibits the phosphorylation of Axin and APC by GSK-3β (17, 29). Furthermore, it has been shown that B56, a subunit of PP2A, binds to APC and that expression of B56 regulates the stability of β-catenin (44). It is intriguing to speculate that Dvl regulates their activities directly in the Axin complex. The third possibility is that Dvl induces a conformational change of Axin by the binding to Axin, thereby inhibiting
to target GSK-3β in close proximity to its substrates in the Axin complex. This is supported by the in vitro observations that Dvl inhibits GSK-3β-dependent phosphorylation of Axin, APC, and β-catenin in the Axin complex, although the bindings of GSK-3β, β-catenin, and APC to Axin are not changed, and that Dvl does not affect GSK-3β activity to phosphorylate the peptide substrate (22, 26). However, it has been recently shown that Dvl induces the dissociation of GSK-3β from Axin homolog in Xenopus embryos (28). The discrepancies between in vitro and embryo observations remain to be clarified.

Since Axam competes with Dvl for the binding to Axin, it may antagonize the action of Dvl, thereby enhancing the phosphorylation by GSK-3β in the Axin complex. As the enhancement of the phosphorylation in the Axin complex leads to the degradation of β-catenin (22–24), it is speculated that Axam degrades β-catenin. Indeed, our results demonstrate that Axam down-regulates β-catenin in SW480 cells. Furthermore, Axam inhibits the axis formation in Xenopus embryos and suppresses the secondary axis formation induced by Xwnt-8 and Dvl-1 but not by β-cateninA5. Therefore, Axam functions between Dvl and β-catenin and promotes degradation of β-catenin.

We have also found that Axam inhibits the formation of head structure of the secondary axis induced by β-cateninA5 although it does not influence the frequency of axis duplication. These results suggest that Axam has an additional function and acts downstream of β-catenin.

Although it has been shown that ectopically expressed Dvl and Axin form a complex with each other in intact cells (25–27), it is hard to show their endogenous interaction (data not shown). This may suggest the presence of the protein that inhibits the complex formation of Dvl and Axin. Based on the present study, we propose that Axam may form a complex with Axin constitutively in the absence of Wnt, thereby Dvl does not bind to Axin. In response to Wnt, the affinity of Dvl for Axin may increase; therefore, it may replace Axam on Axin. In this context, it is known that casein kinases I and II phosphorylate and acts downstream of β-catenin. Indeed, our results demonstrate that Axam has an additional function to target GSK-3β and promotes degradation of β-catenin.

Acknowledgments—We are grateful to Drs. B. Dallapipecola, G. Novelli, D. Kimelman, Y. Matsuura, and M. Nakata for donating plasmids, baculovirus, and antibodies. We also thank the Research Center for Molecular Medicine and Research Facilities for Laboratory Animal Sciences, Hiroshima University School of Medicine, for the use of their facilities.