To understand the mechanism of how Axin acts as an inhibitory molecule in the Wnt pathway, we generated a series of mutated forms of Axin. From the binding experiments, we defined the domains of Axin that bind glycogen synthase kinase-3β (GSK-3β) and β-catenin. We also examined the ability of each Axin mutant to inhibit lymphoid enhancer factor-1 (Lef-1) reporter activity in a cell line expressing high levels of β-catenin. Axin mutants that did not bind GSK-3β or β-catenin were ineffective in suppressing Lef-1 reporter activity. Binding GSK-3β and β-catenin was not sufficient for this inhibitory effect of Axin. Axin mutants with C-terminal truncations lacked the ability to inhibit Lef-1 reporter activity, even though they bound GSK-3β and β-catenin. The C-terminal region was required for binding to Axin itself. Substitution of the C-terminal region with an unrelated dimerizing molecule, the retinoid X receptor restored its inhibitory effect on Lef-1-dependent transcription. The oligomerization of Axin through its C terminus is important for its function in regulation of β-catenin-mediated response.

The Wnt signaling pathway controls developmental processes in both invertebrates and vertebrates (1, 2). Wnt inhibits the activity of glycogen synthase kinase-3β (GSK-3β) by an unknown mechanism, and this inhibition leads to the accumulation of cytoplasmic β-catenin. The protein level of cytoplasmic β-catenin is post-transcriptionally regulated by GSK-3β (3, 4). When GSK-3β activity is inhibited, cytoplasmic β-catenin is stabilized, which allows it to accumulate. This accumulated cytoplasmic β-catenin interacts with the T-cell factor/Lef-1 family of transcription factors and then enters the nucleus to enhance T-cell factor/Lef-1-dependent transcription. The oligomerization of Axin through its C terminus is important for its function in regulation of β-catenin-mediated response.

Experimental Procedures

Cell Culture and Transfection—COS-7 cells and SW480 cells were grown in Dulbecco’s modified Eagle’s medium, 10% fetal calf serum. Transfections were performed by LipofectAMINE™ (Life Technologies, Inc.) following the protocol of the manufacturer.

Plasmid Constructions—All the mouse Axin expression constructs were c-Myc epitope tagged (EQKLISEEDLNEED) and subcloned into pcDNA3.1 vector (Invitrogen). Full-length Axin and was generated as described previously (15). Other truncated mutant constructs were made by restriction enzyme digestion and re-ligation using the adapters, or polymerase chain reaction. The following: AN-Axin digested at Asp1 site (deleted amino acids 126–299), ΔRGS-Axin, digested at first and second PstI sites (deleted amino acids 251–351), ΔNM-Axin, digested at BbrP1 site (deleted amino acids 126–604), ΔMC-Axin, digested at BbrP1 site (deleted amino acids 604–956), ΔAxin, digested at BrbP1 and BbrP1 sites (deleted amino acids 322–602), ΔCM-Axin, digested at BbrP1 and EcoRI sites (deleted amino acids 603–809), ΔC-Axin, digested at EcoRI site (deleted amino acids 805–956), and ΔCC-Axin was made by ligating polymerase chain reaction products (primers: 5′-TCA GCTTCGAATCCGCGAGCGTCATCGA TATCAGCTCCGCCCCCTCTCCGAGGCGCGC TCGCTACAGA TATCCCTTGTTCAGCTCCCTCC’ and 5′-TCCCTCTTTGGCGCG CTCATCGA TATCAGCTCCGCCCCCTCTCCGAGGCGCGC TCGCTACAGA TATCCCTTGTTCAGCTCCCTCC’). The mouse full-length β-catenin cDNA was a generous gift from Dr. A. Nagafuchi (Dept. of Cell Biol., Howard Hughes Medical Institute. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked ‘advertisement’ in accordance with 18 U.S.C. Section 1734 solely to indicate this fact. This paper is available online at http://www.jbc.org

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The abbreviations used are: GSK-3β, glycogen synthase kinase-3β; Lef-1, lymphoid enhancer factor-1; APC, adenomatous polyposis coli; RGS, regulator of G-protein signaling; GST, glutathione S-transferase; RXR, retinoid X receptor; Ecd, ecdysone receptor; PAGE, polyacrylamide gel electrophoresis.

Functional Domains of Axin

IMPORTANCE OF THE C TERMINUS AS AN OLIGOMERIZATION DOMAIN

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labeled Axin (20 μl each) in binding buffer (20 mM Tris-HCl, pH 7.5, 1 mM EDTA, 0.1% Triton X-100, 0.3 M NaCl, and 1 mM phenylmethylsulfonyl fluoride). For β-catenin and Axin binding, GST-β-catenin immobilized to glutathione-Sepharose 4B was mixed with in vitro translated Axin (20 μl each) in binding buffer. For Axin-Axin binding, GST-DN-Axin immobilized to glutathione-Sepharose 4B was mixed with in vitro translated Axin. After incubating at 4 °C for 4 h, the mixtures were extensively washed and in vitro translated Axin bound to immobilized protein was analyzed by SDS-PAGE and autoradiography.

Immunoprecipitation and Immunoblotting—48 h after transfection, cells were lysed in lysis buffer (20 mM Tris-HCl, pH 7.5, 1 mM EDTA, 0.1% Triton X-100, 0.15 M NaCl, and 1 mM phenylmethylsulfonyl fluoride). Cell lysates were incubated with antibodies as indicated at 4 °C overnight, then with Dynabeads coupled to sheep anti-mouse IgG1 (Dynal) for 1 h. Immunocomplexes were washed extensively with lysis buffer and analyzed by immunoblotting. Enhanced chemiluminescence reagents (Amersham) were used for detection of the immunoblots.

Lef-1 Reporter Gene Assay—Lef-1 reporter gene assay was performed as described previously (15). Briefly, SW480 cells were seeded at 1 × 10⁵ cells/well in 12-well culture plate, then transfected on the next day by LipofectAMINE 48 h before the assay. Luciferase activity was measured by luciferase assay system (Promega) and β-galactosidase activity was measured by β-Gal Assay System II (CLONTECH) using Lumino- meter (Monolight 2010, Analytical Luminescence Laboratory). pCG-Lef-1 and pGL3-fo7LEF-luciferase were kindly provided by Dr. R. Grosschedl (University of California, San Francisco). pTK-β-galactosidase was obtained from Promega.

RESULTS AND DISCUSSION

Interactions of Axin Mutants with GSK-3β—Axin was recently identified as a GSK-3β-binding protein by yeast two-hybrid screening (15, 16). To determine the binding domain for GSK-3β in Axin, we made mutant constructs in which small regions of Axin were deleted (Fig. 1). First, we checked the interaction of GSK-3β and in vitro translated Axin constructs (Fig. 2A). We found that the GSK-3β binding domain mapped to the middle part of Axin as reported previously (16–18). The RGS domain and the C terminus region were not required for binding to GSK-3β. The association was also assessed in COS-7 cells. Axin mutants were expressed and immunoprecipitated; the binding of GSK-3β was assessed by immunoblotting (Fig. 3A). Consistent with the in vitro results, the middle region of Axin conferred GSK-3β binding.

Interaction of Axin with β-Catenin—Next, we examined the interaction of β-catenin and Axin deletion mutants. From the in vitro binding experiments using recombinant β-catenin and in vitro translated Axin mutants, the β-catenin binding domain in Axin overlapped with the GSK-3β binding domain but extended further toward the C terminus (Fig. 2B). Neither the RGS domain nor the DIX domain was required for β-catenin binding (Fig. 1).

Bridging Effect of Axin between GSK-3β and β-Catenin—In a previous study (15), we showed that Axin is a bridging molecule between GSK-3β and β-catenin. For example in COS-7 cells or 293 cells, β-catenin and GSK-3β do not form a complex unless Axin is exogenously expressed. We measured this complex formation by detecting GSK-3β in the β-catenin immunocomplex by immunoblotting. Among Axin mutants, FL-, ΔRGS-,
\[ \Delta N, \Delta CM, \Delta C, \text{ and } \Delta CC-Axin provide this bridging function (Fig. 3B). \] These data suggested that the bridging function is mediated through the central region of Axin.

**Inhibition of Lef-1 Transcriptional Activity in Colon Cancer Cells**—Using a Lef-1 reporter gene assay, we have shown that Axin inhibits Lef-1 transcriptional activity in SW480 cells, a colon cancer cell line that carries an APC mutation (15). In this cell line, cytosolic \( \beta \)-catenin is stabilized and accumulates, causing an increase in Lef-1 transcriptional activity (12, 13).

We examined the inhibitory effect of Axin deletion mutants in SW480 cells. FL-, \( \Delta RGS- \), and \( \Delta N \)-Axin showed inhibitory effects in the Lef-1 reporter gene assay, and Axin mutants deficient in the bridging function (\( \Delta NM-, \Delta MC-, \text{ and } \Delta M- \)) did not show any inhibition (Fig. 4). This result suggests that Axin binding to both GSK-3\( \beta \) and \( \beta \)-catenin is necessary for inhibition of Lef-1 reporter gene transcription. This is consistent with the hypothesis that a major effect of Axin is to facilitate GSK-3\( \beta \) mediated phosphorylation of \( \beta \)-catenin (16). However, \( \Delta CM-, \Delta C-, \text{ and } \Delta CC-Axin also lacked the inhibitory effect, even though those mutants can act as bridging molecules between GSK-3\( \beta \) and \( \beta \)-catenin (Fig. 3B). Therefore the bridging effect is not sufficient for Axin to suppress Lef-1-dependent transcription. An additional function of Axin must be provided by its C-terminal region to allow inhibition of Lef-1-dependent transcription.

**The C Terminus Region of Axin Is Required for Its Oligomerization**—Despite their bridging function, Axin mutants with C-terminal deletions did not inhibit Lef-1 reporter activity in SW480 cells (Fig. 4). The C terminus of Axin contains a DIX domain, which shares loose homology with Dishevelled (14, 19). We tested whether Axin binds Dishevelled by overexpressing each protein in COS-7 cells but could not detect any interaction (data not shown). We then examined whether Axin binds to itself using in vitro translated protein. In this assay, recombinant GST-\( \Delta N \)-Axin bound to in vitro translated FL-Axin but not to \( \Delta MC-, \Delta CM-, \Delta C-, \text{ and } \Delta CC-Axin (Fig. 5). \] These data indicate that the C-terminal half of Axin contains a domain that mediates oligomerization. Although binding sites for GSK-3\( \beta \) and \( \beta \)-catenin in Axin overlap, C-terminally deleted Axin mutants, as well as FL-Axin, could bind both molecules simultaneously in vitro (data not shown). Because \( \Delta CM- \) and \( \Delta CC-Axin did not bind to \( \Delta N \)-Axin, both CM region (amino acids 603–809) and DIX domain appeared to be crucial for oligomerization.

**The Dimerized \( \Delta C \)-Axin Restored Its Inhibitory Effect on Lef-1 Reporter Gene Activity**—To test whether oligomerization of Axin is important for its function, we fused \( \Delta C \)-Axin to the RXX, which is known to form a homodimer in vivo (20). \( \Delta C \)-RXX-Axin was able to inhibit Lef-1 reporter gene activity in
SW480 cells (Fig. 6). ΔC-Axin fused to EcR, which does not form a homodimer (21), lacked the inhibitory effect on Lef-1 reporter gene assay. Both ΔC-RXR-Axin and ΔC-EcR-Axin could bind GSK-3β and β-catenin in vivo (data not shown). This result suggest that oligomerization of Axin as well as bridging of GSK-3β and β-catenin is necessary for the function of Axin.

Concluding Discussion—In this study, we identified binding domains of GSK-3β and β-catenin in Axin. We showed that these domains of Axin are important for its inhibition of Lef-1-dependent transcription in colon cancer cells. In Xenopus experiments, ΔRGS-Axin acts as a dominant-negative molecule, which activates the Wnt pathway (14). It was also shown that the RGS domain in Axin binds to APC (17, 22). From our results, the RGS domain is not required for binding to GSK-3β or β-catenin and is not necessary for inhibition of Lef-1 reporter activity in colon cancer cells. It has been suggested that Axin acts as a negative regulatory molecule in the Wnt pathway by binding β-catenin to GSK-3β and APC (30, 34), yet our data using colon cancer cells expressing high levels of β-catenin are necessary for the function of Axin to inhibit Lef-1 reporter activity in vivo.

In this study, we identified binding experiments, ΔGSK-3β acts as a negative regulatory molecule in the Wnt pathway by binding β-catenin to GSK-3β and APC (16). Yet our data using colon cancer cells expressing high levels of β-catenin and GSK-3β as a substrate, which mediates degradation of β-catenin (16). Yet our data using colon cancer cells expressing high levels of β-catenin are not consistent with this simplistic model, because we found Axin mutants that link β-catenin and GSK-3β but do not inhibit Lef-1 reporter activity. β-catenin is already stabilized independent of GSK-3β activity in those cancer cells. Our results suggest that besides scaffolding a kinase and a substrate Axin has some other effects that appear to depend on the C terminus of the molecule. There are mutant mice of Axin that have a dominant kinked tail phenotype (23). In those mice, Axin gene products are likely to have a deletion at the C terminus as a result of transposon insertion. This also suggests that the C terminus of Axin has some functional importance. We showed that the C terminus region of Axin is involved in the oligomerization of Axin. The chimeric ΔC-Axin construct fused to the RRX, which mediates dimerization inhibited Lef-1 reporter activity. These data suggest that both oligomerization and bridging of GSK-3β with β-catenin are necessary for the function of Axin to inhibit Lef-1-dependent transcription.

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