Axin, a Negative Regulator of the Wnt Signaling Pathway, Directly Interacts with Adenomatous Polyposis Coli and Regulates the Stabilization of \(\beta\)-Catenin

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The regulators of G protein signaling (RGS) domain of Axin, a negative regulator of the Wnt signaling pathway, made a complex with full-length adenomatous polyposis coli (APC) in COS, 293, and L cells but not with truncated APC in SW480 or DLD-1 cells. The RGS domain directly interacted with the region containing the 20-amino acid repeats but not with that containing the 15-amino acid repeats of APC, although both regions are known to bind to \(\beta\)-catenin. In the region containing seven 20-amino acid repeats, the region containing the latter five repeats bound to the RGS domain of Axin. Axin and \(\beta\)-catenin simultaneously interacted with APC. Furthermore, Axin stimulated the degradation of \(\beta\)-catenin in COS cells. Taken together with our recent observations that Axin directly interacts with glycogen synthase kinase-3\(\beta\) (GSK-3\(\beta\)) and \(\beta\)-catenin and that it promotes GSK-3\(\beta\)-dependent phosphorylation of \(\beta\)-catenin, these results suggest that Axin, APC, GSK-3\(\beta\), and \(\beta\)-catenin make a tetrameric complex, resulting in the regulation of the stabilization of \(\beta\)-catenin.

Axin, which is a product of the mouse Fused locus, has been identified as a negative regulator of the Wnt signaling pathway (1). Fused is a mutation that causes dominant skeletal and neurological defects and recessive lethal embryonic defects including neuroectodermal abnormalities (2–4). Because dorsal injection of wild-type Axin in Xenopus embryos blocks axis formation and coinjection of Axin inhibits Wnt\(\beta\)-, Dsh-, and kinase-negative GSK-3\(\beta\)-induced axis duplication (1), Axin could exert its effects on axis formation by inhibiting the Wnt signaling pathway. However, the molecular mechanism by which Axin regulates axis formation has not been shown. We have recently identified rat Axin (rAxin) as a GSK-3\(\beta\)-interacting protein (5). rAxin is phosphorylated by GSK-3\(\beta\), directly binds to not only GSK-3\(\beta\) but also \(\beta\)-catenin, and promotes GSK-3\(\beta\)-dependent phosphorylation of \(\beta\)-catenin (5). Because the phosphorylation of \(\beta\)-catenin by GSK-3\(\beta\) is essential for the down-regulation of \(\beta\)-catenin (6, 7), our results suggest that rAxin may induce the degradation of \(\beta\)-catenin. These actions of rAxin are consistent with the observation that Axin inhibits dorsal axis formation in Xenopus embryos, because the accumulation of \(\beta\)-catenin induces the axis duplication (8).

It has been shown that besides the phosphorylation by GSK-3\(\beta\), the down-regulation of \(\beta\)-catenin requires APC, which is a tumor suppressor linked to FAP and to the initiation of sporadic human colorectal cancer (9). The middle portion of APC contains three successive 15-amino acid (aa) repeats followed by seven related but distinct 20-aa repeats. Both types of repeats are able to bind independently to \(\beta\)-catenin (10–12). In FAP and colorectal cancers, most patients carry APC mutations that result in the expression of truncated proteins (9). Almost all mutant proteins lack the C-terminal half including most of the 20-aa repeats but retain the 15-aa repeats. Colorectal carcinoma cells with mutant APC contain large amounts of monomeric \(\beta\)-catenin (13). The accumulated \(\beta\)-catenin translocates to the nucleus, and this translocation involves the association of \(\beta\)-catenin with the transcription enhancers of the lymphocyte enhancer binding factor/T cell factor family (14, 15). Because the APC mutants retain the \(\beta\)-catenin-binding activity, the interaction of APC with \(\beta\)-catenin is not sufficient for the down-regulation of \(\beta\)-catenin. How APC down-regulates \(\beta\)-catenin and the relationship between APC and Axin in the degradation of \(\beta\)-catenin are not clear.

In addition to GSK-3\(\beta\)- and \(\beta\)-catenin-binding sites, rAxin has a domain that is homologous to RGS, and this domain is called the RGS domain (1, 5). RGS has been originally identified as a protein that binds to the GTP- but not GDP-bound form of G\(a\) and stimulates GTP hydrolysis of G\(a\) (16). It has been shown that \(\Delta RGS\), a mutant of Axin in which the RGS domain is deleted, acts as a potent dorsalizer, producing a secondary axis and that Axin blocks the axis-inducing activity of \(\Delta RGS\) (1). These results indicate that \(\Delta RGS\) acts through a dominant-negative mechanism to inhibit an endogenous Axin activity and that it competes for binding to a protein with which Axin normally interacts. Therefore, the RGS domain may have an activity to transmit the signal by interacting with other protein(s). Here we report that the RGS domain of rAxin directly interacts with the region containing the 20-aa repeats of APC and that rAxin stimulates the down-regulation of \(\beta\)-catenin. Taken together with our recent observations (5), these results indicate that Axin directly binds to APC, \(\beta\)-catenin, and GSK-3\(\beta\) and that it regulates the stabilization of \(\beta\)-catenin.

**EXPERIMENTAL PROCEDURES**

**Materials and Chemicals**—APC cDNA, 293 cells, L cells, and SW480 and DLD-1 cells were kindly supplied from Drs. T. Akiyama (Osaka University, Suita, Japan), K. Morishita (Daichi Pharmaceutical Co. Ltd., Tokyo, Japan), A. Nagafuchi and Sh. Tsukita (Kyoto University, Kyoto, Japan), and E. Tahara (Hiroshima University, Hiroshima, Japan), respectively. GST and MBP fusion proteins were purified from Escherichia coli according to the manufacturer’s instructions. The anti-
APC (Ab-1) and β-catenin antibodies were purchased from Oncogene Science Inc. (Cambridge, MA) and Transduction Laboratories (Lexington, KY), respectively. [35S]Methionine and [35S]Cysteine were purchased from Amersham Inc. (Buckinghamshire, United Kingdom). Other materials and chemicals were from commercial sources.

**Plasmid Constructions**—pEF-BOS-Myc/rAxin (full-length), pBSKS/rAxin (full-length), pBJ-Myc/rAxin-(1–229) (lane 1), pEF-BOS/Myc/rAxin-(1–713), pBJ-Myc/rAxin-(298–713), pEF-BOS/Myc/rAxin-(298–506), pBJ-Myc/rAxin-(713–832), pGEX-2T-β-catenin, and pMAL-c2(rAxin-(298–506)–506) were constructed as described (5). To construct pGEX-2T/RGS, the RGS cDNA fragment encoding rAxin-(89–216) was synthesized by polymerase chain reaction and inserted into pGEX-2T. To construct pMAL-c2 containing APC mutants, pMIKtwo/AvP was digested with various restriction enzymes, and the APC cDNA fragments were inserted into pMAL-c2. These procedures will be described in detail elsewhere. To construct pMAL-c2(rAxin-(57–832)), pBSKS/rAxin was digested with Smal and EcoRV, and the rAxin cDNA fragment was inserted into pMAL-c2, which was digested with XbaI and blunted with Klenow fragment. To construct pGEX-2T(rAxin-(1–529)), pBSKS/rAxin was digested with Smal and PvuII, and this fragment was inserted into Smal cut pGEX-2T. To construct pCGEN/β-catenin, pBSKS/β-catenin was digested with XhoI, blunted with Klenow fragment, and digested with XbaI. The β-catenin cDNA fragment was inserted into pCGN.

**Interaction of APC with rAxin**—COS cells (10-cm diameter dish) transfected with pβ-J and pEF-BOS-derived plasmids were lysed as described (17–19). rAxin and its deletion mutants were tagged with Myc epitope at their N termini. The lysates (160–800 μg of protein) were immunoprecipitated with the anti-Myc antibody, then the precipitates were probed with the anti-APC and β-catenin antibodies. When the interaction of the RGS domain of rAxin with APC was examined in vitro, 1 μg GST-RGS was incubated with the lysates (200 μg of protein) of COS, 293, L, SW480, and DLD-1 cells for 2 h at 4 °C. GST-RGS were precipitated with glutathione-Sepharose 4B, and the precipitates were probed with the anti-APC antibody.

**Kinetics of the Binding of rAxin, β-Catenin, and APC**—Various deletion mutants of MBP-APC (0.5–10 pmol) immobilized on the amyllose resin were incubated with various concentrations of GST-RGS, GST-rAxin-(1–529), and GST-β-catenin in 100 μl of reaction mixture (20 mM Tris/HCl (pH 7.5) and 1 mM dithiothreitol) for 2 h at 4 °C. MBP fusion proteins were precipitated by centrifugation, and the precipitates were probed with the anti-GST antibody. When the effect of rAxin on the interaction of APC with β-catenin was examined, 50 nm GST-β-catenin was incubated with 250 nm MBP-APC-(959–1338) in the presence of various concentrations of MBP-rAxin-(298–506) or MBP-APC (full-length) in 100 μl of reaction mixture for 2 h at 4 °C. GST-β-catenin was precipitated by glutathione-Sepharose 4B, and the precipitates were probed with the anti-MBP antibody. Where specified, the relative intensities of the precipitated GST and MBP fusion proteins were quantitated by densitometric tracing of the stained sheets using an NIH image program.

**Pulse-Chase Analysis of β-Catenin—**COS cells (60–70% confluent on a 35-mm diameter dish) were transfected with pCGN/β-catenin alone or with pCGN/β-catenin and pEF-BOS-Myc/rAxin (full-length). After 60 h, pulse-chase analysis was performed as described (13). Briefly, the cells were pulse-labeled with [35S]methionine and [35S]cysteine (50 μCi/ml) for 30 min at 37 °C. Then the cells were lysed immediately or at the indicated times following incubation with excess unlabeled methionine and cysteine. The lysates were immunoprecipitated with the anti-HA antibody, and the precipitates were probed with the anti-HA antibody and analyzed with a Fuji BAS 2000 image analyzer.

**RESULTS AND DISCUSSION**

**Complex Formation of the RGS Domain of rAxin with APC in Intact Cells**—We have recently found that rAxin directly binds to β-catenin (5). Because it has been shown that β-catenin directly binds to APC (10–12), we examined whether rAxin makes a complex with APC through β-catenin. Various deletion mutants of Myc-rAxin expressed in COS cells were immunoprecipitated with the anti-Myc antibody. Consistent with our recent observations (5), β-catenin was coprecipitated with Myc-rAxin (full-length), Myc-rAxin-(1–713), Myc-rAxin-(298–713), and Myc-rAxin-(298–506) (Fig. 1A). Among them, APC was detected in the Myc-rAxin (full-length) and Myc-rAxin-(1–713) immune complexes, but not in the Myc-rAxin-(298–713) and Myc-rAxin-(298–506) immune complexes (Fig. 1A). Unexpectedly, APC but not β-catenin was detected in the Myc-rAxin-(1–229) immune complex. Neither β-catenin nor APC was coprecipitated with Myc-rAxin-(713–832). Because rAxin-(1–229) contains the RGS domain, we examined whether the RGS domain itself (amino acids 89–216) makes a complex with APC. APC in COS, 293, and L cells were coprecipitated with GST-RGS (Fig. 1B). It is known that APC is truncated at amino acids 1337 and 1427 in SW480 and DLD-1 cells, respectively, and that these truncated forms of APC fail to down-regulate β-catenin (9, 13). These APC mutants in SW480 and DLD-1 cells were not coprecipitated with GST-RGS (Fig. 1B). Consistent with the previous observations (10, 11), both full-length and truncated APC were coprecipitated with GST-β-catenin (Fig. 1B). These results suggest that the RGS domain of rAxin makes a complex with the C-terminal half of APC in intact cells. Taken together with our observations (5), rAxin has distinct binding sites for APC, β-catenin, and GSK-3β. It is notable that the APC mutants in colorectal carcinoma cell lines such as SW480 and DLD-1 cells do not associate with rAxin.

**Direct Interaction of rAxin with APC**—To examine whether the RGS domain of rAxin directly interacts with APC, various deletion mutants of APC were purified as MBP fusion proteins (Fig. 2A). GST-RGS bound to MBP-APC-(1211–2075), which contains seven 20-aa repeats, in a dose-dependent manner (Fig. 2B). The K_{d} value was calculated to be 115 nM. However, GST-RGS did not bind to MBP-APC-(959–1338) which contains three 15-aa repeats and the first 20-aa repeat (Fig. 2B). These results show that the RGS domain of rAxin directly interacts with the region containing the 20-aa repeats of APC. To characterize the interaction of APC with rAxin further, MBP-APC-(1211–1787), which contains the former four 20-aa repeats, and MBP-APC-(1788–2075), which contains the latter...
Fig. 2. Direct interaction of rAxin with APC. A, deletion mutants of APC. The arrows mark the points of APC truncations in SW480 and DLD-1 cells. B, interaction of the RGS domain with the 20-aa repeats of APC. MBP-APC-(1211–2075) (0.5 pmol) (○) or MBP-APC-(959–1338) (5 pmol) (□) immobilized on the amylose resin was incubated with the indicated concentrations of GST-RGS. MBP fusion proteins were precipitated by centrifugation, and the amounts of interacted GST-RGS were quantified by densitometric tracing. C, binding site of APC for the RGS domain. MBP-APC-(1211–1787) (lane 1), MBP-APC-(1788–2075) (lane 2), MBP-APC-(1211–1495) (lane 3), or MBP-APC-(1475–1787) (lane 4) (5 pmol each) immobilized on the amylose resin was incubated with 1 µM GST-RGS and GST-β-catenin. MBP fusion proteins were precipitated by centrifugation, and the precipitates were probed with the anti-GST antibody. The arrow and arrowhead indicate the positions of GST-β-catenin and GST-RGS, respectively. The results shown are representative of three independent experiments.

three 20-aa repeats, were purified. Both GST-RGS and GST-β-catenin bound to MBP-APC-(1211–1787), but they bound to MBP-APC-(1788–2075) less efficiently (Fig. 2C). Furthermore, GST-β-catenin bound to both MBP-APC-(1211–1495) and MBP-APC-(1475–1787), whereas GST-RGS bound to MBP-APC-(1475–1787) but not to MBP-APC-(1211–1495) (Fig. 2C). Therefore, the RGS domain does not interact with the region of APC containing the 15-aa repeats and the first and the second 20-aa repeats, which binds to β-catenin. These results are consistent with the observations that β-catenin but not the RGS domain of rAxin associated with the APC mutants in SW480 and DLD-1 cells.

A family of RGS proteins has been identified in eukaryotic species ranging from yeast to mammals (16). The three-dimen-
sional structure of a stable complex of RGS4 and Goα1 has been determined (20). Residues that form the hydrophobic core of the RGS box of RGS4 are well conserved in the RGS domain of rAxin. However, 11 residues of RGS4 that make direct contact with Goα1 are not conserved in the RGS domain of rAxin except for one amino acid. Therefore, it is conceivable that the RGS domain interacts with the proteins other than the α subunit of G proteins. Our results are the first demonstration that a member of the RGS protein family has a binding partner other than the α subunit of G proteins.

Effect of rAxin on the Interaction of APC with β-catenin—We have found that rAxin-(298–506) directly binds to β-catenin-(175–423), which contains armadillo repeats 2–7 (5). APC interacts with the armadillo repeats 2–10 of β-catenin (12). Therefore, we next examined whether rAxin and APC share the binding site on β-catenin. GST-β-catenin bound to MBP-rAxin-(298–506) and MBP-APC-(959–1338) in a dose-dependent manner, and their Kd values were calculated to be 227 nM and 273 nM, respectively (data not shown). MBP-rAxin-(298–506) inhibited the binding of MBP-APC-(959–1338) to GST-β-catenin in a dose-dependent manner (Fig. 3A). MBP-rAxin (full-length) also inhibited their binding although the inhibitory efficiency was less than MBP-APC-(959–1338) (Fig. 3A). Furthermore, we examined the effect of rAxin-(1–529), which contains the binding sites for APC and β-catenin, on the interaction of β-catenin with MBP-APC-(1211–1787), which binds to both β-catenin and rAxin. Although GST-rAxin-(1–529) bound to MBP-APC-(1211–1787) in a dose-dependent manner, it did not affect significantly the interaction of GST-β-catenin with MBP-APC-(1211–1787) (Fig. 3B). These results are consistent with the results that in APC-(1211–1787) β-catenin prefers APC-(1211–1495) to APC-(1475–1787); inversely the RGS domain binds to APC-(1475–1787) but not to APC-(1211–1495). Taken together, although the RGS-catenin-binding sites of rAxin and APC do not simultaneously bind to β-catenin, β-catenin does not compete with rAxin for the binding to APC when they are full-length proteins. Furthermore, since rAxin has distinct binding sites for APC and β-catenin, these three proteins could make a complex.

It has been shown that APC down-regulates the level of β-catenin (9, 13). This APC activity was localized to the central region of the protein which contains at least three of the 20-aa...
repeat sequence (9). The fragment containing the 15-aa repeats and the first 20-aa repeat does not down-regulate β-catenin (9).

Our results indicate that this region binds to β-catenin but not to the RGS domain of rAxin. Therefore, the binding of APC to β-catenin is not sufficient for decreasing the β-catenin level, and the binding to Axin may be necessary.

Down-regulation of β-Catenin by rAxin—To investigate whether rAxin regulates the stabilization of β-catenin, pulse-chase analysis in COS cells expressing HA-β-catenin was performed. Although equivalent amounts of HA-β-catenin were immunoprecipitated with the anti-HA antibody from the lysates of COS cells expressing HA-β-catenin alone and coexpressing HA-β-catenin and Myc-rAxin as assessed by immunoblot analysis (data not shown), pulse-labeled HA-β-catenin gradually decreased with a half-life of 4 h (Fig. 4). When Myc-rAxin was cotransfected, HA-β-catenin exhibited a shorter half-life (Fig. 4). These results indicate that rAxin has an activity to stimulate the down-regulation of β-catenin.

To down-regulate β-catenin, its phosphorylation by GSK-3β is required, and the mutations of the phosphorylation site stabilize β-catenin (7). It has been reported recently that β-catenin is ubiquitinated and that the ubiquitination of β-catenin is abolished when the GSK-3β phosphorylation site in β-catenin is mutated (21). Therefore, the degradation of β-catenin could be regulated by the ubiquitination-proteasome pathway. Taken together with our observations that rAxin promotes GSK-3β-dependent phosphorylation of β-catenin (5), the present results strongly suggest that rAxin stimulates the down-regulation of β-catenin in cooperation with APC. Literally hundreds of APC mutants have been reported in FAP and cancer patients (9). Almost all of these mutations are confined to the 5’-half of the APC coding sequence and result in truncation of APC, which lacks the C-terminal half containing most of the 20-aa repeats. Our results indicate that these APC mutants do not interact with rAxin. Therefore, the reason why mutations of APC cause cancer may be due to its inability to bind to Axin.

It has been reported that there are mutations of serine in consensus sequence of the phosphorylation site of β-catenin for GSK-3β in melanoma and colon cancer that have normal APC protein (22–24). Thus, there are at least two ways to increase levels of β-catenin due to mutations in APC and β-catenin itself. Therefore, mutations in APC, GSK-3β-, and β-catenin-binding sites on Axin may cause human cancer.

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Fig. 4. Pulse-chase analysis of β-catenin. COS cells expressing HA-β-catenin alone (●) or HA-β-catenin with Myc-rAxin (○) were pulse-labeled with [35S]methionine and [35S]cysteine for 30 min and lysed immediately (time 0) or at the indicated periods of time following incubation with excess unlabeled methionine and cysteine. HA-β-catenin was immunoprecipitated with the anti-HA antibody, and the incorporation of [35S] into HA-β-catenin was immunoprecipitated with the anti-HA antibody, and the incorporation of [35S] into HA-β-catenin was measured. Although equivalent amounts of HA-β-catenin were immunoprecipitated, HA-β-catenin was cotransfected. HA-β-catenin was analyzed with a Fuji BAS 2000 image analyzer and expressed as the percentage of the value of time 0. The results shown are means ± S.D. of five independent experiments.