Axin, a negative regulator of the Wnt signaling pathway, forms a complex with GSK-3β and β-catenin and promotes GSK-3β-dependent phosphorylation of β-catenin

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Glycogen synthase kinase-3 (GSK-3) mediates epidermal growth factor, insulin and Wnt signals to various downstream events such as glycogen metabolism, gene expression, proliferation and differentiation. We have isolated here a GSK-3β-interacting protein from a rat brain cDNA library using a yeast two-hybrid method. This protein consists of 832 amino acids and possesses Regulators of G protein Signaling (RGS) and dishevelled (Dsh) homologous domains in its N- and C-terminal regions, respectively. The predicted amino acid sequence of this GSK-3β-interacting protein shows 94% identity with mouse Axin, which recently has been identified as a negative regulator of the Wnt signaling pathway; therefore, we termed this protein rAxin (rat Axin). rAxin interacted directly with, and was phosphorylated by, GSK-3β. rAxin also interacted directly with the armadillo repeats of β-catenin. The binding site of rAxin for GSK-3β was distinct from the β-catenin-binding site, and these three proteins formed a ternary complex. Furthermore, rAxin promoted GSK-3β-dependent phosphorylation of β-catenin. These results suggest that rAxin negatively regulates the Wnt signaling pathway by interacting with GSK-3β and β-catenin and mediating the signal from GSK-3β to β-catenin.

Keywords: Axin/β-catenin/GSK-3β phosphorylation

Introduction

Glycogen synthase kinase-3 (GSK-3) was originally characterized as a serine/threonine kinase that phosphorylates and inactivates glycogen synthase, and subsequently was demonstrated to be identical to protein kinase F3 that activates ATP-Mg-dependent type-1 protein phosphatase (Plyte et al., 1992). GSK-3 is now implicated in the regulation of several physiological responses in mammalian cells by phosphorylating many substrates including neuronal cell adhesion molecule, neurofilament, synapsin 1, tau, transcription factors and adenomatous polyposis coli (APC) gene product (Mandelkow et al., 1992; Plyte et al., 1992; Yang et al., 1992; Rubinfeld et al., 1996). The cDNAs of GSK-3α and GSK-3β in mammals have been isolated and they encode the protein kinases with Mr of 51 and 47 kDa, respectively (Woodgett, 1990). Mammalian GSK-3β is structurally and functionally homologous to the Drosophila zeste-white3/shaggy gene product (Ruel et al., 1993). The shaggy gene product has been found to be required at several developmental stages during fruit fly embryogenesis for correct embryogenic segmentation (Simpson et al., 1988; Perrimon and Smouse, 1989). In Saccharomyces cerevisiae, the MCK1 and MDS1 genes encode serine/threonine kinases which are homologous to mammalian GSK-3 and to the Drosophila shaggy gene product. The MCK1 and MDS1 gene products play a role in the chromosomal segregation processes (Puziss et al., 1994). In Schizosaccharomyces pombe, the skp1+ gene product is a homolog of GSK-3 and regulates cytokinesis (Plyte et al., 1996). Xenopus GSK-3 has been shown to regulate ventral differentiation during early Xenopus development (He et al., 1995). Furthermore, the Dictyostelium homolog of GSK-3 (GSKA) has been found to be important for cellular differentiation (Harwood et al., 1995). Thus, GSK-3 is highly conserved throughout evolution and plays a fundamental role in cellular responses.

Genetic evidence reveals that Drosophila wingless signals through an intracellular cascade that includes dishevelled (Dsh), shaggy and armadillo, a β-catenin homolog (Miller and Moon, 1996; Nusse, 1997). In the absence of a wingless signal, shaggy antagonizes downstream elements of the wingless pathway via changes in the level of armadillo and, in response to wingless signaling, there is a decrease in the phosphorylation of armadillo and an increase in its stability. In Xenopus, interference with GSK-3 induces an ectopic dorsal axis which is identical to that resulting from overexpression of Wnt and β-catenin (He et al., 1995). In mammals, it has been shown also that GSK-3 is inactivated by Wnt, and that β-catenin accumulates in the cytoplasm following the Wnt signals and translocates to the nucleus (Polakis, 1997). This translocation involves the association of β-catenin with the transcriptional enhancers of lymphocyte enhancer binding factor/T cell factor (LEF/TCF) family (Behrens et al., 1996; Molenaar et al., 1996). A dominant-negative form of Xenopus TCF-3 inhibits axis formation (Molenaar et al., 1996). Furthermore, it has been shown that GSK-3β phosphorylates APC and that the phosphorylation enhances the binding of APC to β-catenin (Rubinfeld et al., 1996). It is well known that APC is required for the degradation of β-catenin, although the role of APC is not well understood (Polakis, 1997). Thus, the Wnt signaling pathway through GSK-3β regulates the determination of cell fate. In specifying cell fate, GSK-3β may interact with several cellular targets. Apart from the demonstration of GSK-3β binding to APC–β-catenin complex (Rubinfeld et al., 1996), GSK-3β may have other binding partners as well.
β-Catenin has a consensus sequence phosphorylation site for GSK-3β, and GSK-3β acts to cause the degradation of β-catenin (Miller and Moon, 1996). Elimination of the possible phosphorylation site increases the stability of β-catenin (Munemitsu et al., 1996; Yost et al., 1996). It has been shown recently that the ubiquitination–proteasome pathway is involved in the degradation of β-catenin and that mutations in the GSK-3β consensus phosphorylation site of β-catenin prevent ubiquitination (Aberle et al., 1997). Thus, it appears that GSK-3β phosphorylates β-catenin and that the phosphorylation of β-catenin is essential for its degradation. Indeed, it has been reported...
that in *Xenopus*, GSK-3β phosphorylates β-catenin *in vitro* (Yost *et al.*, 1996). However, it has been shown that mammalian GSK-3β does not significantly phosphorylate β-catenin *in vitro* (Rubinfeld *et al.*, 1996). These results suggest that the factor(s) involved in the phosphorylation of β-catenin may be lost.

To obtain further insights into the action of GSK-3β on the degradation of β-catenin, we have tried to find a GSK-3β-interacting protein using a yeast two-hybrid method. From a rat brain cDNA library, we have isolated a protein which interacts with GSK-3β, and we found that this protein shared 94% identity with mouse Axin (Zeng *et al.*, 1997), indicating that it is rat Axin (rAxin). Axin has been found to regulate negatively a critical step in the formation of the embryonic axis and exert its effects at a very early stage by specifically inhibiting the Wnt signaling pathway (Zeng *et al.*, 1997). We show here that rAxin is phosphorylated by GSK-3β and that it directly interacts with both GSK-3β and β-catenin. Furthermore, we demonstrate that rAxin promotes GSK-3β-dependent phosphorylation of β-catenin. These results suggest that Axin negatively regulates the Wnt signaling pathway by interacting with GSK-3β and β-catenin and mediating the signal from GSK-3β to β-catenin.

**Results**

**Isolation of GSK-3β-interacting protein**

To identify proteins that physically interact with GSK-3β, we conducted a rat brain cDNA library screening by the yeast two-hybrid method. From 3.7×10⁶ initial transformants, four clones were found to confer both the His⁺ and LacZ⁺ phenotypes on L40 containing pBTM116HA/H11001 and pBTM116HA/H11003, encoding rAxin (rat Axin) and Dsh. Therefore, we tried to characterize this clone and did not analyze the other clones further here.

A full-length cDNA of this GSK-3β-interacting protein was isolated from a rat brain cDNA library. This clone spanned a distance of 3.5 kb and contained an uninterrupted ORF of 2496 bp, encoding a predicted protein of 832 amino acids with a calculated Mr of 92 855 Da (Figure 1A). The first ATG was preceded by stop codons in all three reading frames, and the 5′-non-coding region had a high percentage of GC base pairs (81%). The neighboring sequence of the second ATG was more consistent than that of the first with the translation initiation start proposed by Kozak (1987), but we could not determine which is the first Met. When we isolated this cDNA, besides dbEST sequences (DDBJ/EMBL/GenBank Accession nos: AA198606, AA028403, AA238939, W97777, AA259709 and AA170717), no protein closely related to this GSK-3β-interacting protein was identified. However, during preparation of this manuscript, this protein was found to share 94% amino acid identity with mouse Axin (Zeng *et al.*, 1997), so we designated this GSK-3β-interacting protein rAxin (rat Axin). Although it appears that the ORF of mouse Axin extends upstream of rAxin, it has been suggested that the first or second Met of mouse Axin, which is in the same position as that of rAxin, could serve as an initiation codon (Zeng *et al.*, 1997).

There are alternative splicing products, termed form 1 and 2, in mouse Axin. A 36 amino acid segment is inserted in form 2. The rAxin that we isolated is form 1, since 36 amino acids with a calculated Mr of 92 855 Da (Figure 1A). The full-length cDNA insert was found to encode a sequence containing a long open reading frame (ORF) and the consensus sequence for a stop codon. Further, the protein encoded by this cDNA shared high homology with Regulators of G protein Signaling (RGS) and Dsh. Therefore, we tried to characterize this clone and did not analyze the other clones further here.

A full-length cDNA of this GSK-3β-interacting protein was isolated from a rat brain cDNA library. This clone spanned a distance of 3.5 kb and contained an uninterrupted ORF of 2496 bp, encoding a predicted protein of 832 amino acids with a calculated Mr of 92 855 Da (Figure 1A). The first ATG was preceded by stop codons in all three reading frames, and the 5′-non-coding region had a high percentage of GC base pairs (81%). The neighboring sequence of the second ATG was more consistent than that of the first with the translation initiation start proposed by Kozak (1987), but we could not determine which is the first Met. When we isolated this cDNA, besides dbEST sequences (DDBJ/EMBL/GenBank Accession nos: AA198606, AA028403, AA238939, W97777, AA259709 and AA170717), no protein closely related to this GSK-3β-interacting protein was identified. However, during preparation of this manuscript, this protein was found to share 94% amino acid identity with mouse Axin (Zeng *et al.*, 1997), so we designated this GSK-3β-interacting protein rAxin (rat Axin). Although it appears that the ORF of mouse Axin extends upstream of rAxin, it has been suggested that the first or second Met of mouse Axin, which is in the same position as that of rAxin, could serve as an initiation codon (Zeng *et al.*, 1997). There are alternative splicing products, termed form 1 and 2, in mouse Axin. A 36 amino acid segment is inserted in form 2. The rAxin that we isolated is form 1, since 36 amino acids which correspond to mouse Axin (860–895) are lacking. The N-terminal region of rAxin, residues 89–216, shared 31% amino acid identity with residues 58–
Interaction of rAxin with GSK-3β

To examine whether rAxin interacts with GSK-3β in intact cells, we co-expressed rAxin with GSK-3β in COS cells (Figure 2A). rAxin and GSK-3β were tagged with Myc and hemagglutinin 1 (HA) epitopes, respectively, at their N-termini. HA-GSK-3β and endogenous GSK-3β were detected by the anti-GSK-3β antibody. When the lysates co-expressing Myc-rAxin with HA-GSK-3β were immunoprecipitated with the anti-Myc antibody, HA-GSK-3β was detected in the Myc-rAxin immune complex (Figure 2B, lanes 1–3). Similarly, when the same lysates were immunoprecipitated with the anti-HA antibody, Myc-rAxin was detected in the HA-GSK-3β immune complex (Figure 2B, lanes 4–6). Neither Myc-rAxin nor HA-GSK-3β was immunoprecipitated from the lysates expressing both proteins with non-immune immunoglobulin (data not shown). To show that Myc-rAxin interacts with endogenous GSK-3α and GSK-3β, the lysates expressing Myc-rAxin alone were immunoprecipitated with the anti-Myc antibody. Endogenous GSK-3α and GSK-3β were co-precipitated with Myc-rAxin (Figure 2C). These results suggest that rAxin forms a complex with GSK-3α and GSK-3β in intact cells. HA-GSK-3βK85M and HA-GSK-3βY216F, in which ATP binding and tyrosine phosphorylation sites respectively are mutated, were expressed in COS cells. Neither HA-GSK-3βK85M nor HA-GSK-3βY216F showed kinase activities for GSK peptide 1 (data not shown). Co-transfection of Myc-rAxin with these HA-GSK-3β mutants did not alter the level of expression of transfected Myc-rAxin as assessed by immunoblot analysis (data not shown). When these lysates were immunoprecipitated with the anti-HA antibody, Myc-rAxin was not co-precipitated with HA-GSK-3βK85M or HA-GSK-3βY216F (Figure 2D). These results suggest that the kinase activity of GSK-3β may be necessary for its complex formation with rAxin.

To determine which region of rAxin interacts with GSK-3β, various deletion mutants of Myc-rAxin were expressed in COS cells (Figure 3A and B, upper panel).
immunoprecipitated with the anti-Myc antibody, endogenous GSK-3β was co-precipitated with Myc-rAxin (full-length), Myc-rAxin-(298–832), Myc-rAxin-(1–713), Myc-rAxin-(298–713) and Myc-rAxin-(298–506), but not with Myc-rAxin-(508–832), Myc-rAxin-(713–832) or Myc-rAxin-(1–229) (Figure 3B, lower panel). These results indicate that the region containing residues 298–506 of rAxin is sufficient and necessary for its complex formation with GSK-3β.

**Phosphorylation of rAxin by GSK-3β**

To determine whether rAxin is a substrate for GSK-3β, Myc-rAxin immunoprecipitated from the COS cell lysates was incubated with or without glutathione-S-transferase (GST) fused to GSK-3β (GST–GSK-3β). rAxin was phosphorylated without GST–GSK-3β, and this phosphorylation was enhanced by GST–GSK-3β (Figure 4A). GST–GSK-3β phosphorylated Myc-rAxin in a time- and dose-dependent manner (Figure 4B and C). Approximately 3–4 mol of phosphate was maximally incorporated into 1 mol of Myc-rAxin. In Figure 2C, we show that endogenous GSK-3β is co-precipitated with Myc-rAxin. Therefore, the phosphorylation of Myc-rAxin without GST–GSK-3β might be due to the associated endogenous GSK-3β. However, we cannot rule out the possibility that GSK-3β phosphorylates and activates other protein kinases which interact with and phosphorylate Myc-rAxin. Alternatively, other protein kinases associated with Myc-rAxin might phosphorylate it independently of GSK-3β.

To determine which regions of rAxin were phosphorylated by GSK-3β, various deletion mutants of Myc-rAxin described in Figure 3A were immunoprecipitated from the COS cell lysates. Myc-rAxin (full-length), Myc-rAxin-(298–832), Myc-rAxin-(1–713), Myc-rAxin-(298–713) and Myc-rAxin-(298–506) were phosphorylated by GST–GSK-3β (Figure 4D, lanes 1, 2 and 6–8); however, Myc-rAxin-(508–832), Myc-rAxin-(713–832) and Myc-rAxin-(1–229) were not (lanes 3–5). These results indicate that Myc-rAxin-(298–506) contains at least one phosphorylation site for GSK-3β. Taken together with the observations in Figure 3, the physical interaction of rAxin with GSK-3β may be required for the phosphorylation of rAxin.

To examine whether endogenous Axin interacts with GSK-3β in intact cells, the COS cell lysates were immunoprecipitated with the anti-GSK-3β antibody, and the immunoprecipitates were incubated in the kinase reaction mixture. The protein with an Mr of 105 kDa, which is similar to the Mr of Myc-rAxin, was phosphorylated, but this phosphorylated protein was not observed in the immunoprecipitates with non-immunoglobulin (Figure 4E). These results suggest that endogenous Axin forms a complex with and is phosphorylated by GSK-3β.

**Kinetics of the phosphorylation of rAxin-(298–506) by GSK-3β and the interaction of rAxin-(298–506) with GSK-3β**

To rule out the involvement of endogenous GSK-3β in the phosphorylation of and interaction with rAxin, we purified rAxin-(298–506) from *Escherichia coli* as a maltose-binding protein (MBP) fusion protein. GST–GSK-3β phosphorylated MBP–rAxin-(298–506) in a time-dependent manner (Figures 4F and 5A). The *Kₐ₅* value for the phosphorylation of MBP–rAxin-(298–506) by GST–GSK-3β was calculated to be 93 nM. The sequence S/TXXXS/T is known to be a consensus sequence for a phosphorylation site (Plyte *et al.*, 1992). In residues 298–506 of rAxin, there are three possible phosphorylation sites: SANDSEQQS³³⁰, SĐĐT/LSLT³⁴¹ and SLTD⁵. All serine and threonine residues of these possible phosphorylation sites were substituted by alanine, and the phosphorylation of these mutants by GST–GSK-3β was examined. The phosphorylation levels of MBP–rAxin-(298–506), MBP–rAxin-(298–506, 322/326/330A), MBP–rAxin-(298–506, 333/337/341A) and MBP–rAxin-(298–506, 339/343A) by GST–GSK-3β were reduced to 55–60% of wild-type (Figure 4F). These results indicate that rAxin is directly phosphorylated by GSK-3β and that it has multiple phosphorylation sites for GSK-3β.

**Fig. 3.** Interacting domain of rAxin with GSK-3β. (A) Deletion mutants of rAxin. The hatched and white boxes indicate the RGS homologous and Dsh homologous domains, respectively. (B) Expression of rAxin deletion mutants and their interaction with GSK-3β. The lysates of COS cells expressing Myc-rAxin (full-length) (lane 1), Myc-rAxin-(298–832) (lane 2), Myc-rAxin-(508–832) (lane 3), Myc-rAxin-(713–832) (lane 4), Myc-rAxin-(1–229) (lane 5), Myc-rAxin-(1–713) (lane 6), Myc-rAxin-(298–713) (lane 7) and Myc-rAxin-(298–506) (lane 8) were immunoprecipitated with the anti-Myc antibody, then the precipitates were probed with the anti-Myc antibody (upper panel) or the anti-GSK-3β antibody (lower panel). IP, immunoprecipitation; Ab, antibody; Ig, immunoglobulin. The arrowhead indicates the position of endogenous GSK-3β.
Fig. 4. Phosphorylation of rAxin by GSK-3β. (A) Autoradiography. Myc-rAxin (full-length) immunoprecipitated from the COS cell lysates (100 μg of protein) was incubated with (lane 2) or without (lane 1) GST–GSK-3β (100 ng of protein) for 10 min, then the samples were subjected to SDS–PAGE followed by autoradiography. The arrowhead and arrow indicate the positions of Myc-rAxin and GST–GSK-3β, respectively. (B) Time course. Myc-rAxin immunoprecipitated from the COS cell lysates was incubated with (●) or without (○) GST–GSK-3β (100 ng of protein) for the indicated periods of time. The radioactivity of the phosphorylated Myc-rAxin was counted. (C) Dose dependency. Myc-rAxin immunoprecipitated from the COS cell lysates was incubated with the indicated amounts of GST–GSK-3β for 5 min. (D) Phosphorylation of rAxin mutants by GSK-3β. Myc-rAxin (full-length) (lane 1), Myc-rAxin-(298–832) (lane 2), Myc-rAxin-(508–832) (lane 3), Myc-rAxin-(713–832) (lane 4), Myc-rAxin-(1–229) (lane 5), Myc-rAxin-(1–713) (lane 6), Myc-rAxin-(298–713) (lane 7) and Myc-rAxin-(298–506) (lane 8) immunoprecipitated from the COS cell lysates were incubated with GST–GSK-3β (100 ng of protein) for 30 min. (E) Co-precipitation of endogenous Axin with GSK-3β. The lysates (200 μg of protein) of COS cells (lanes 2 and 3) were immunoprecipitated with the anti-GSK-3β antibody (lane 2) or non-immune immunoglobulin (lane 3). The immunoprecipitates were incubated in the kinase reaction mixture. The lysates of COS cells expressing Myc-rAxin (lane 1) were used to determine the position of the phosphorylated Myc-rAxin. IP, immunoprecipitation; Ab, antibody; NI, non-immune immunoglobulin. (F) Phosphorylation sites of rAxin for GSK-3β. MBP–rAxin-(298–506) (lane 2), MBP–rAxin-(298–506, 322/326/330A) (lane 3), MBP–rAxin-(298–506, 333/337/341A) (lane 4) and MBP–rAxin-(298–506, 339/343A) (lane 5) (375 ng of protein each) were incubated with GST–GSK-3β (100 ng of protein) for 15 min. As a control, MBP–rAxin-(298–506) was incubated without GST–GSK-3β (lane 1). WT, MBP–rAxin-(298–506). The arrowhead indicates the positions of MBP–rAxin-(298–506) and its mutants. The results shown are representative of three independent experiments.

MBP–rAxin-(298–506) bound to GST–GSK-3β in a dose-dependent manner, and the K_d value of the binding was ~65 nM (Figure 5B). These results indicate that rAxin directly interacts with GSK-3β. Neither GST–GSK-3βK85M nor GST–GSK-3βY216F exhibited kinase activities for GSK peptide 1 (data not shown). Consistent with the results in Figure 2D, MBP–rAxin-(298–506) did not bind to these GSK-3β mutants (Figure 5B). Since ATP was not used in this experiment, it is suggested that the interaction of rAxin with GSK-3β does not require the phosphorylation of rAxin but that the interaction may require the structure of GSK-3β which is catalytically active.

Interaction of rAxin with β-catenin
It has been demonstrated that Axin negatively regulates the Wnt signaling pathway either at the level of GSK-3
Complex formation of Axin with GSK-3β and β-catenin

Fig. 5. Kinetics of the phosphorylation of rAxin-(298–506) by GSK-3β and the interaction of rAxin-(298–506) with GSK-3β. (A) Direct phosphorylation of rAxin-(298–506) by GSK-3β. MBP–rAxin-(298–506) (1 μg of protein) was incubated with (○) or without (○) GST–GSK-3β (100 ng of protein) for the indicated time. (B) Direct binding of rAxin-(298–506) to GSK-3β. GST–GSK-3β (2 μmol), GST–GSK-3βK85M (●) and GST–GSK-3βY216F (▼) (2.2 pmol each) immobilized to glutathione–Sepharose 4B were incubated with the indicated concentrations of MBP–rAxin-(298–506) for 2 h. After GST–GSK-3β and its mutants were precipitated by centrifugation, the precipitates were probed with the anti-MBP antibody and the amounts of interacted MBP–rAxin-(298–506) were quantified by densitometric tracing. The results shown are representative of three independent experiments.

Fig. 6. Interaction of rAxin with β-catenin. (A) Interaction of rAxin with β-catenin in intact cells. The lysates (20 μg of protein) of COS cells expressing Myc-rAxin (lanes 1 and 3) or control cells (lanes 2 and 4) were probed with the anti-Myc and endogenous β-catenin antibodies (lanes 1 and 2), and the same lysates (200 μg of protein) were immunoprecipitated with the anti-Myc antibody, then probed with the anti-Myc and β-catenin antibodies (lanes 3 and 4). (B) Interaction of the N-terminal region of β-catenin with rAxin. GST–β-catenin (lane 1), GST–β-catenin(1–423) (lane 2), GST–β-catenin(423–781) (lane 3) (9 pmol each) were subjected to SDS–PAGE followed by Coomassie Brilliant Blue staining. After the lysates (200 μg of protein) of COS cells expressing Myc-rAxin were incubated with GST–β-catenin (lane 4), GST–β-catenin(1–423) (lane 5) and GST–β-catenin(423–781) (lane 6) (18 pmol each), GST–β-catenin and its deletion mutants were precipitated with glutathione–Sepharose 4B. The precipitates were probed with the anti-Myc antibody. (C) Interacting domain of rAxin with β-catenin. After the lysates of COS cells expressing various deletion mutants of Myc-rAxin described in Figure 3A were immunoprecipitated with the anti-Myc antibody, the precipitates were probed with the anti-β-catenin antibody. (D) Direct interaction of rAxin with β-catenin. After GST–β-catenin-(1–423) (lane 1), GST–β-catenin-(1–131) (lane 2), GST–β-catenin-(132–423) (lane 3), GST–β-catenin-(175–423) (lane 4) and GST–β-catenin(423–781) (lane 5) (18 pmol each) were incubated with MBP–rAxin-(298–506) (60 pmol) for 2 h, GST–β-catenin deletion mutants were precipitated with glutathione–Sepharose 4B. The precipitates were probed with the anti-MBP antibody. IP, immunoprecipitation; Ab, antibody. The large arrows, arrowheads and small arrow indicate the positions of Myc-rAxin, endogenous β-catenin and MBP–rAxin-(298–506), respectively. The results shown are representative of four independent experiments.

or further downstream of GSK-3, and that it acts upstream of β-catenin (Zeng et al., 1997). Therefore, we next examined the relationship between rAxin and β-catenin. β-Catenin was expressed in COS cells (Figure 6A). When the lysates expressing Myc-rAxin were immunoprecipitated with the anti-Myc antibody, endogenous β-catenin was co-precipitated with Myc-rAxin (Figure 6A). Neither Myc-rAxin nor β-catenin were immunoprecipitated from the lysates expressing Myc-rAxin with non-immune immunoglobulin (data not shown). Myc-rAxin was co-precipitated with GST–β-catenin-(1–423) but not with GST–β-catenin-(423–781) from the COS cell lysates (Figure 6B). These results suggest that rAxin forms a complex with the N-terminal region of β-catenin in intact cells. To determine which region of rAxin binds to β-catenin, various deletion mutants of Myc-rAxin described in Figure 3A were expressed in COS cells, and the lysates were immunoprecipitated with the anti-Myc antibody. Endogenous β-catenin was co-precipitated with Myc-rAxin (full-length), Myc-rAxin-(298–832), Myc-rAxin(1–713), Myc-rAxin(298–713) and Myc-rAxin(298–506), but not with Myc-rAxin-(508–832), Myc-rAxin(713–832) or Myc-rAxin(1–229) (Figure 6C). These results indicate that the region containing residues 298–506 of rAxin is sufficient and necessary for it to form a complex with β-catenin. To examine whether β-catenin...
Table I. Two-hybrid interactions between β-catenin and rAxin

<table>
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<th>Growth on selective media (–His)</th>
<th>β-Galactosidase activity</th>
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<tr>
<td></td>
<td>pGAD</td>
<td>pGAD/β-catenin</td>
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<tr>
<td>pBTM116HA</td>
<td>–</td>
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<td>pBTM116HA/rAxin</td>
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L40 was co-transformed with pBTM116HA or pBTM116HA/rAxin and pGAD or pGAD/β-catenin. To assay for protein interaction-induced growth, cells were streaked on selective medium (–His) containing 3-aminotriazole and incubated for 5 days at 25°C. To assay for β-galactosidase activity, cells were streaked on permissive medium (+His) and incubated for 3 days at 30°C, then β-galactosidase activity was measured. (–), negative; (+), positive.

directly binds to rAxin and which region of β-catenin is involved in this binding, various deletion mutants of β-catenin were purified from *E. coli* as GST fusion proteins. GST–β-catenin-(1–423), GST–β-catenin-(132–423) and GST–β-catenin-(175–423), but not GST–β-catenin-(1–131) or GST–β-catenin-(423–781), bound to MBP–rAxin (298–506) (Figure 6D). GST–β-catenin-(132–423) contains seven armadillo repeats, and β-catenin-(175–423) lacks the first armadillo repeat. The N-terminal region containing the first armadillo repeat of β-catenin is known to be involved in its interaction with α-catenin (Hülsken et al., 1994; Funayama et al., 1995). Therefore, these results demonstrate that rAxin directly interacts with the armadillo repeats 2–7 of β-catenin and that it does not compete with α-catenin for binding to β-catenin. The interaction of rAxin with β-catenin was confirmed by the yeast two-hybrid method (Table I).

**Effect of the phosphorylation of rAxin on its interaction with GSK-3β and β-catenin**

From the results of Figures 3–6, we found that rAxin-(298–506) is phosphorylated by GSK-3β and binds to both GSK-3β and β-catenin. We therefore examined whether the phosphorylation of rAxin affects its interaction with GSK-3β and β-catenin. When MBP–rAxin-(298–506) was incubated with GST–GSK-3β in the presence or absence of ATP, GST–GSK-3β bound to the phosphorylated and non-phosphorylated MBP–rAxin-(298–506) with similar efficiency (Figure 7A). GST–β-catenin also bound to the phosphorylated and non-phosphorylated MBP–rAxin-(298–506) without any significant difference (Figure 7B). These results indicate that the phosphorylation of rAxin does not affect its affinity for GSK-3β and β-catenin.

**Ternary complex formation of GSK-3β, rAxin and β-catenin**

The observations that rAxin directly binds to both GSK-3β and β-catenin prompted us to examine whether these three proteins form a ternary complex. When Myc-rAxin and HA-GSK-3β were co-expressed in COS cells and HA-GSK-3β was immunoprecipitated with the anti-HA antibody, both Myc-rAxin and endogenous β-catenin were detected in the HA-GSK-3β immune complex (Figure 8A, lanes 1 and 4). However, in the absence of Myc-rAxin, β-catenin was not detected in the HA-GSK-3β immune complex (Figure 8A, lanes 2 and 5). Furthermore, Myc-rAxin–endogenous GSK-3β complex was co-precipitated with GST–β-catenin, and inversely Myc-rAxin–endogenous β-catenin complex was co-precipitated with GST–GSK-3β (Figure 8B). These results suggest that GSK-3β, rAxin and β-catenin form a ternary complex in intact cells and that GSK-3β and β-catenin interact with separate sites on rAxin. To investigate this possibility, two deletion mutants, MBP–rAxin-(353–437) and MBP–rAxin-(437–506), were purified from *E. coli*. GST–GSK-3β bound to MBP–rAxin-(298–506) and MBP–rAxin-(353–437) but not to MBP–rAxin-(437–506) (Figure 8C). On the other hand, GST–β-catenin bound to MBP–rAxin-(298–506) and MBP–rAxin-(437–506) but not to MBP–rAxin-(353–437) (Figure 8C). These results clearly indicate that the binding site of rAxin for GSK-3β is distinct from the β-catenin-binding site.

**Phosphorylation of β-catenin by GSK-3β in the presence of rAxin**

It has been shown that β-catenin has a consensus sequence phosphorylation site for GSK-3β and that β-catenin mutants lacking this site are more stable than wild-type (Munemitsu et al., 1996; Yost et al., 1996). Therefore, it is thought that the phosphorylation of β-catenin by GSK-3β regulates the stabilization of β-catenin. However, it has been reported that mammalian GSK-3β does not
phosphorylate β-catenin significantly in vitro (Rubinfeld et al., 1996). Therefore, we examined whether GSK-3β phosphorylates β-catenin in the conditions under which these proteins form a complex with rAxin. In the absence of MBP–rAxin-(298–506), no significant phosphorylation of GST–β-catenin by GST–GSK-3β was observed, whereas in its presence the phosphorylation was greatly increased (Figure 9A and D). MBP–rAxin-(298–506) enhanced GST–β-catenin phosphorylation by GST–GSK-3β in a dose-dependent manner (Figure 9B and E). When the amount of GST–β-catenin was increased, its phosphorylation level was also increased (Figure 9C and F). Thus, it appears that rAxin promotes GSK-3β-dependent phosphorylation of β-catenin. Furthermore, GST–β-catenin-(1–423) but not GST–β-catenin-(423–781) was phosphorylated by GST–GSK-3β in the presence of rAxin-(298–506) (data not shown). These results were consistent with the observations that GST–β-catenin-(1–423) interacted with rAxin and that it has a consensus sequence phosphorylation site for GSK-3β. Since MBP–rAxin-(298–506) does not increase the phosphorylation of GSK peptide 1 by GST–GSK-3β (data not shown), it is unlikely that rAxin activates GSK-3β kinase activity. Taken together, these results suggest that rAxin plays a role in placing β-catenin in the vicinity of GSK-3β, thereby enhancing the phosphorylation of β-catenin by GSK-3β.

Discussion

We have isolated a protein which interacts with GSK-3β and have determined its primary structure. Since this protein has been found to be 94% identical with mouse Axin (Zeng et al., 1997), we have named it rAxin. Axin is the product of the Fused locus (Zeng et al., 1997). It has been found that the mouse mutant of Fused carries recessive mutations that are lethal at E8–E10, and that a remarkable property of embryos homozygous for this mutant is a duplication of the embryo axis (Perry et al., 1995). When Axin mRNA is injected into Xenopus embryos, most embryos develop with strong axial defects (Zeng et al., 1997). Co-expression of Axin inhibits the induction of the secondary dorsal axis by Wnt, Dsh and kinase-negative GSK-3, while it does not affect β-catenin-and Siamois-induced secondary axis formation. Although injection of Noggin or dominant-negative bone morphogenetic protein receptor also causes secondary axis formation, Axin fails to block this induction (Zeng et al., 1997). These results indicate that Axin exerts its function on axis formation by specifically inhibiting the Wnt pathway. However, no biochemical information has been provided on how and with which components of the Wnt pathway it might interact.

We have shown that GSK-3β interacts with rAxin in COS cells and that residues 353–437 of rAxin are responsible for the interaction. This association has been observed with wild-type but not with catalytically inactive mutants of GSK-3β, GSK-3βY845F and GSK-3βY216F. It has been reported that insect cell-expressed GSK-3βY216F showed 10% activity of the wild-type (Hughes et al., 1993), but our results have shown that neither HA-GSK-3βY216F in COS cells nor bacterially expressed GST–GSK-3βY216F exhibit kinase activities. Although we do not know the reasons why GSK-3βY216F kinase activities are different
Fig. 9. Phosphorylation of β-catenin by GSK-3β in the presence of rAxin. (A and D) GST–β-catenin (4 μg of protein) was incubated with the indicated amounts of GST–GSK-3β in the presence (+, ○) or absence (−, □) of MBP–rAxin-(298–506) (200 ng of protein) for 30 min. (B and E) GST–β-catenin (2 μg of protein) was incubated with GST–GSK-3β (400 ng of protein) in the presence of the indicated amounts of MBP–rAxin-(298–506). (C and F) The indicated amounts of GST–β-catenin were incubated with GST–GSK-3β (400 ng of protein) in the presence of MBP–rAxin-(298–506) (200 ng of protein). (A, B and C) Autoradiography. (D, E and F) The radioactivity incorporated into GST–β-catenin was counted. The arrows, large arrowheads and small arrowheads indicate the positions of GST–β-catenin, MBP–rAxin-(298–506) and GST–GSK-3β, respectively. The results shown are representative of four independent experiments.

among expression systems, our results suggest that GSK-3β without kinase activity does not have the ability to bind to rAxin. It is known that recombinant GSK-3β has a specific activity less than that of mammalian expressed protein (Hughes et al., 1993). Our in vitro experiments have shown that GST–GSK-3β binds to MBP–rAxin-(298–506). These results suggest that the interaction of GSK-3β with rAxin requires its kinase activity and that even a low specific kinase activity is sufficient for the interaction. Alternatively, the interaction of GSK-3β with rAxin may require the structure of GSK-3β which is catalytically active rather than the kinase activity itself, and K85 and Y216 may be important for keeping the rAxin-interacting domain of GSK-3β in a binding conformation. We have also demonstrated that rAxin is phosphorylated by GST–GSK-3β and that rAxin-(298–506) contains at least one phosphorylation site for GST–GSK-3β. Since we have used immunoprecipitated rAxin as a GST–GSK-3β substrate, we cannot rule out the possibility that rAxin is co-precipitated with other protein kinases and that the protein kinases are activated by GSK-3β and phosphorylate rAxin. However, MBP–rAxin-(298–506) is phosphorylated by GST–GSK-3β, and mutations in the consensus sequence phosphorylation sites for GSK-3β reduce the phosphorylation of MBP–rAxin-(298–506) by GST–GSK-3β. Therefore, it is likely that GSK-3β directly phosphorylates rAxin and that SANDSEQQS^{330}, SDADTLSLT^{341} and SLTDS^{343} are the phosphorylation sites for GSK-3β. These results are consistent with the observations that 3–4 mol of phosphate are incorporated into 1 mol of immunoprecipitated Myc-rAxin. Of course, it remains to be clarified whether protein kinases other than GSK-3β interact with and phosphorylate rAxin. Although prior phosphorylation of a proto-substrate by a
distinct protein kinase is required for the phosphorylation by GSK-3, in several substrates, such as glycogen synthase, inhibitor-2, CREB and APC (Plyte et al., 1992; Fiol et al., 1994; Rubinfeld et al., 1996), the phosphorylation of rAxin by GSK-3β does not require prior phosphorylation.

Our results have shown that rAxin interacts not only with GSK-3β but also with β-catenin, and that residues 437–506 of rAxin are responsible for its binding to β-catenin. The primary structure of β-catenin consists of an N-terminal portion of ~130 amino acids, a central region of 550 amino acids that contains 12 repeats of 42 amino acids known as armadillo repeats, and a C-terminal region of 110 amino acids (Huber et al., 1997). We have demonstrated that rAxin interacts directly with the region containing armadillo repeats 2–7 of β-catenin. The armadillo repeat region of β-catenin has been shown to bind to α-catenin, cadherin, APC and LEF/TCF (Hülsken et al., 1994; Funayama et al., 1995; Behrens et al., 1996; Molenaar et al., 1996; Polakis, 1997). It remains to be clarified whether rAxin and these proteins share a common binding site on β-catenin. It is unlikely that rAxin does not compete with α-catenin for binding to β-catenin, since the N-terminal region containing the first armadillo repeat of β-catenin is involved in its interaction with α-catenin (Hülsken et al., 1994; Funayama et al., 1995). It has been reported that neither β-catenin(1–423) nor β-catenin(423–781) interact independently with APC (Rubinfeld et al., 1995). Therefore, the Axin-binding site of β-catenin may be distinct from the binding site for APC. It has been shown that APC forms a complex with β-catenin and that the phosphorylation of APC by GSK-3β increases the binding of APC to β-catenin (Rubinfeld et al., 1996). However, the phosphorylation of rAxin by GSK-3β does not affect its binding to GSK-3β and β-catenin. Therefore, we do not know the physiological significance of the phosphorylation of rAxin by GSK-3β.

We have demonstrated that rAxin, GSK-3β and β-catenin form a ternary complex in COS cells. Consistent with these observations, GSK-3β and β-catenin bind to separate sites on rAxin, and these sites are adjacent. Furthermore, we have found that rAxin promotes GSK-3β-dependent phosphorylation of β-catenin. Approximately 0.2–0.3 mol of phosphate was incorporated into 1 mol of GST-β-catenin in the presence of MBP-rAxin (298–506). These results are the first demonstration of the mechanism by which GSK-3β phosphorylates β-catenin efficiently. It is well known that the phosphorylation of β-catenin is essential for its degradation process (Miller and Moon, 1996). Therefore, Axin may cause β-catenin degradation by interacting with both GSK-3β and β-catenin and enhancing the phosphorylation of β-catenin. This model is consistent with the observations that Axin negatively regulates the Wnt signaling pathway in development of Xenopus embryos (Zeng et al., 1997). Although it is not known whether APC promotes GSK-3β-dependent phosphorylation of β-catenin, APC and Axin may regulate β-catenin degradation by different mechanisms.

rAxin possesses an RGS homologous domain. Recently, a family of RGS proteins has been identified in eukaryotic species ranging from yeast to mammals (Dohlman and Thorner, 1997). The RGS domain of members of this family binds to the GTP- but not GDP-bound form of Go and stimulates GTP hydrolysis of Go. Although there is no direct evidence showing that G protein is involved in the Wnt signaling pathway, it is intriguing to speculate that the RGS domain of Axin is a functional Go GAP and that Axin thereby inhibits the Wnt signaling pathway. The three-dimensional structure of a stable complex of RGS4 and Go has been determined (Tesmer et al., 1997). 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 Gi1α are not conserved in the RGS domain of rAxin, with the exception of one amino acid. Indeed, our preliminary experiments demonstrate that the RGS domain of rAxin neither stimulates the GTP hydrolysis of Gi2α nor binds to the GDP-bound form of Gi2z (unpublished data). So far, all of the RGS proteins currently characterized bind to members of the Giα class of G protein α subunits, including Goα, Gzα and Giα. Therefore, the RGS domain of rAxin may regulate the G protein activity of members other than the Giα class. Alternatively, it may have an additional activity of transmitting the signal by interacting with other protein(s). It has been shown that ΔRGS, a mutant of Axin in which the RGS domain is deleted, acts as a potent dorsailizer, producing a secondary axis, and that Axin blocks the axis-inducing activity of ΔRGS (Zeng et al., 1997). These results indicate that ΔRGS 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.

Our observation that the RGS domain is distinct from the GSK-3β- and β-catenin-binding sites of rAxin is consistent with these results. The RGS domain of rAxin could be involved in the degradation of β-catenin. In addition to the RGS domain, rAxin has a domain homologous to the N-terminal region of Dsh. Since the function of this region of Dsh is not known, the role of the Dsh homologous domain in the action of rAxin remains to be clarified.

It has been demonstrated that Axin negatively regulates the Wnt signaling either at the level of GSK-3 or further downstream, and that it acts upstream of β-catenin (Zeng et al., 1997). Three possible mechanisms of the mode of action of Axin have been suggested: (i) Axin increases the level of the phosphorylation of GSK-3 substrates by inhibiting the protein dephosphorylation; (ii) Axin stimulates GSK-3 activity; or (iii) the RGS domain of Axin acts to inhibit the Wnt signaling pathway, probably by inhibiting the G protein functions. In addition to these possibilities, our observations demonstrated here suggest that the interaction of Axin with GSK-3β and β-catenin promotes GSK-3β-dependent phosphorylation of β-catenin, thereby leading to the degradation of β-catenin. Although our experiments were performed in COS cells that overexpressed rAxin and in vitro, it is possible that the interactions that we observed in COS cells are representative of intact cells physiologically expressing Axin.

Materials and methods

Materials and chemicals

Yeast strain L40, plasmid vectors for two-hybrid screening, a pGAD10-derived rat brain cDNA library, pBSSK/GSK-3β, pBSSK/β-catenin, pGAD/β-catenin, a λZAP rat brain cDNA library, pEF-BOS, pGEX-KG, a peptide substrate of GSK-3 (GSK peptide 1), the anti-HA antibody

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and the anti-MBP antibody were kindly supplied by Drs Y.Takai and K.Tanaka (Osaka University, Suita, Japan), J.R. Woodgett (Ontario Cancer Institute, Toronto, Canada), A.Nagafuchi and S.Tsunaka (Kyoto University, Kyoto, Japan), S.Kuroda and K-Kataoka (Science and Technology, Ikoma, Japan), Y.Hata (ERATO, Japan Science and Technology Corp., Kobe, Japan), S.Nagata (Osaka University, Suita, Japan) (Mizushima and Nagata, 1990), F.Tanamoni (University of California, Los Angeles, CA), C.W.Tureck (University of California, San Francisco, CA) (Murai et al., 1996), Q.Hu (Chiron Corp., CA) and M.Kusumi (Suumotomo Electric Industries, Yokohama, Japan), respectively. The anti-Myc antibody was prepared from 9E10 cells. GST–GSK-3β was purified from E.coli as described (Murai et al., 1996). GST and MBP fusion proteins were purified from E.coli according to the manufacturer’s instructions. The anti-GSK-3β and β-catenin antibodies were purchased from Transduction Laboratories (Lexington, KY), the anti-GSK-3β-antibody was fromUBI (Lake Placid, NY) [α-32P]ATP and [γ-32P]ATP were obtained from Amersham Inc. (Buckinghamshire, UK). Other materials were from commercial sources.

### Plasmid construction

To construct pBTM116HA/GSK-3β, pBSKSK/GSK-3β was digested with BcII and EcoRI, and blunted with Klenow fragment. The 1.3 kb fragment was inserted into pBTM116HA which was digested with BamHI and blunted with Klenow fragment. To construct pBSKSK/εxin, the 0.2 kb fragment encoding rAxin-(1–70) with a Smal site at the 5′ end was synthesized by PCR. This fragment was digested with Smal and BgII and inserted into the Smal- and BgII-cut pBSKSK containing the 3.2 kb fragment encoding rAxin prepared from the library in pGAD10. pBSKSK/εxin was digested with BgIII and XbaI, and the Smal- and EcoRI encoding rAxin was inserted into pMAL-c2 which was digested with Xbal and blunted with Klenow fragment to construct pMAL-c2/rAxin. To construct pBTM116HA/rAxin, pMAL-c2/rAxin was digested with EcoRI and SalI, and the 3.2 kb fragment encoding rAxin was inserted into the EcoRI- and SalI-cut pBTM116HA. The 1.3 fragments encoding GSK-3βK85M and GSK-3βH11032 were digested with XhoI and Smal and synthesized by PCR and inserted into the Smal- and EcoRI sites synthesized for PCR. The 1.0 kb fragment encoding rAxin-(298–506) in which Ser322, Ser326 and Ser330 were substituted by Ala, was digested with XhoI and blunted with Klenow fragment, and the 2.7 kb fragment was inserted into the Smal-cut pGEX-2T. To construct pMAL-c2/rAxin-(1–423), pBSKSK/β-catenin was digested with BamHI and EcoRI, and the 1.3 kb fragment encoding β-catenin-(1–423) was inserted into the pGEX-2T-cut pGEX-2T. To construct pMAL-c2/rAxin-(423–781), pBSKSK/β-catenin was digested with EcoRI and Xhol, and the 1.4 kb fragment encoding β-catenin-(423–781) was inserted into the EcoRI- and Xhol-cut pGEX-KG. To construct pGEX-2T/β-catenin-(131–157), pGEX-2T/β-catenin-(131–243) and pGEX-2T/β-catenin-(157–243) the fragments encoding β-catenin-(131–157), β-catenin-(132–243) and β-catenin-(157–243) with BamHI and EcoRI sites were synthesized by PCR, digested with BamHI and EcoRI, and inserted into the BamHI- and EcoRI-cut pGEX-2T.

### Yeast two-hybrid screening

Yeast strain L40 (MATa trpl1 leu1 his3 ade2 lys2:LEU4-HIS3 URA3:: -lexa-lacZ) was used as a host for the two-hybrid screening (Noytek et al., 1997). To strain L40 carrying the pGAD10 vector, pBSKSK/εxin was digested with Xbal and blunted with Klenow fragment to construct pGAD-εxin. The fragment encoding rAxin was inserted into pBSKSK/εxin in which the 3.7 kb fragment encoding rAxin was inserted into the SacI-cut pUC19 to construct pUC19/rAxin (298–832). The 1.7 kb fragment encoding rAxin (298–832) was inserted into the SacI-cut pUC19 to construct pUC19/rAxin (298–832). pUC19/rAxin (298–832) was digested with EcoRI and Smal and blunted with Klenow fragment, and the 1.7 kb fragment encoding rAxin (298–832) was inserted into the pGEX-2T-cut pGEX-KG, and the 1.4 kb fragment encoding rAxin-(423–781) was inserted into the EcoRI- and Xhol-cut pGEX-KG. To construct pGEX-2T/β-catenin-(131–157), pGEX-2T/β-catenin-(132–243) and pGEX-2T/β-catenin-(157–243) the fragments encoding β-catenin-(131–157), β-catenin-(132–243) and β-catenin-(157–243) were synthesized by PCR, digested with BamHI and EcoRI, and inserted into the BamHI- and EcoRI-cut pGEX-2T.

### Molecular cloning of rAxin

To obtain full-length cDNA of GSK-3β-interacting protein, the clone isolated by the yeast two-hybrid method was labeled with random primers and [α-32P]dCTP and used to screen a λZAP rabbit brain cDNA library. A number of positive clones were isolated and all clones, collectively spanning 3.5 kb, were sequenced using double-stranded templates, a Thermo Sequenase pre-mixed cycle sequencing kit (Amersham Inc., Buckinghamshire, UK) and a Hitachi DNA sequencer SQ-5500 (Hitachi Ltd., Tokyo, Japan).

### Interaction of GSK-3β, GSK-3α and β-catenin with rAxin in COS cells

COS cells (10 cm diameter dish) transfected with pCGN, pBI- and pEF-BOS-derived plasmids were lysed as described (Kikuchi and Williams, 1996). rAxin and its deletion mutants were tagged with Myc epitope at their N-termini. GSK-3β and its mutants were tagged with HA epitope at their N-termini. The lysates (50–800 μg of protein) were immunoprecipitated with the anti-Myc or HA antibody, then the precipitates were probed with the anti-Myc, HA, GSK-3β, GSK-3α and
β-catenin antibodies (Hinoi et al., 1996; Kikuchi and Williams, 1996; Murai et al., 1997).

Interaction of GST-3β and β-catenin with rAxin in vitro
To determine the Kd value of rAxin binding to GST-3β, GST–GST-3β (2.2 pmol) immobilized to glutathione–Sepharose 4B was incubated with various concentrations of MBP–rAxin (298–506) for 2 h at 4°C in a 40 μl reaction mixture [20 mM Tris–HCl pH 7.5 and 1 mM dithiothreitol (DTT)]. GST–GST-3β was precipitated by centrifugation and the precipitates were probed with the anti-MBP antibody. The relative intensity of precipitated MBP–rAxin (298–506) was quantitated by densitometric tracing of the stained sheets using the NIH image program. When the interaction of β-catenin and GST-3β with rAxin was examined, the wild-type and various mutants of GST–GST-3β or GST–β-catenin were incubated with various mutants of MBP–rAxin in 40 μl reaction mixture or with the lysates (200 μg of protein) of COS cells expressing Myc-rAxin for 2 h at 4°C. GST–β-catenin, GST–GST-3β and their mutants were precipitated with glutathione–Sepharose 4B, then the precipitates were probed with the anti-MBP, Myc, β-catenin and GST-3β antibodies.

GST-3β kinase assay
The kinase activities of GST-3β and its mutants for GST peptide 1 were measured as described (Murai et al., 1996). The lysates (150 μg of protein) of COS cells (6 cm diameter dish) expressing wild-type and mutated HA-GST-3β were immunoprecipitated with the anti-HA antibody. The precipitates were incubated with 50 μM GST peptide 1 in 30 μl of kinase reaction mixture [50 mM Tris–HCl pH 7.5, 10 mM MgCl2, 1 mM DTT and 50 μM [γ-32P]ATP (500–2000 c.p.m./pmol)] for 10 min at 30°C. The reaction mixture was then spotted onto phosphocellulose filters (Whatman PS1) and washed with phosphoric acid (Murai et al., 1996). When the immunoprecipitated Myc-rAxin was used as a substrate, the lysates (50–500 μg of protein) of COS cells expressing wild-type and various Myc-rAxin deletion mutants were immunoprecipitated with the anti-Myc antibody. The precipitates were washed and incubated with GST–GST-3β (100 ng of protein) in 30 μl of the kinase reaction mixture for 30 min at 30°C. When the phosphorylation of rAxin (492–506) and β-catenin by GST–GST-3β was examined, MBP–rAxin (298–506) and GST–β-catenin purified from E.coli were used as substrates. The samples were subjected to SDS-PAGE followed by autoradiography. Where specified, the radioactivity of the phosphorylated rAxin and β-catenin was counted. To examine the effect of the phosphorylation of rAxin on its binding to GST-3β, MBP–rAxin (298–506) (15 pmol) immobilized to amyllose resin was incubated with various concentrations of GST–GST-3β with or without 50 μM ATP in 30 μl of kinase reaction mixture for 30 min at 30°C. MBP–rAxin (298–506) was precipitated by centrifugation, and the precipitates were probed with the anti-GST antibody. To examine the effect of the phosphorylation of rAxin on its binding to β-catenin, MBP–rAxin (298–506) (15 pmol) immobilized to amyllose resin was incubated with GST–GST-3β (6 pmol) with or without 50 μM ATP in 30 μl of kinase reaction mixture for 30 min at 30°C. MBP–rAxin (298–506) was precipitated by centrifugation and the precipitates were washed to remove free GST–GST-3β and ATP. After the precipitates were incubated with various amounts of GST–β-catenin in 30 μl of reaction mixture for 1 h at 4°C, the concentrations of GST–β-catenin bound to the phosphorylated and non-phosphorylated MBP–rAxin (298–506) immobilized to amyllose resin were detected with the anti-GST antibody.

Other assays
Northern blot analysis was performed as described (Maniatis, 1982). Protein concentrations were determined with bovine serum albumin as a standard (Bradford, 1976).

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