Axin, a negative regulator of the Wnt signaling pathway, forms a complex with GSK-3 β and β -catenin and promotes GSK-3 β -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-3 β -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-3 β -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-3 β . rAxin also interacted directly with the armadillo repeats of β -catenin. The binding site of rAxin for GSK-3 β was distinct from the β -catenin-binding site, and these three proteins formed a ternary complex. Furthermore, rAxin promoted GSK-3 β -dependent phosphorylation of β -catenin. These results suggest that rAxin negatively regulates the Wnt signaling pathway by interacting with GSK-3 β and β -catenin and mediating the signal from GSK-3 β to β -catenin.

Keywords: Axin/ β -catenin/GSK-3 β /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 F_A 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 I, 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-3 α and GSK-3 β in mammals have been isolated and they encode the protein kinases with M_rs of 51 and 47 kDa, respectively (Woodgett, 1990).

Mammalian GSK-3 β 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, although the mechanism is not clear (Cook *et al.*, 1996), 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-3 β 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-3 β regulates the determination of cell fate. In specifying cell fate, GSK-3 β may interact with several cellular targets. Apart from the demonstration of GSK-3 β binding to APC- β -catenin complex (Rubinfeld *et al.*, 1996), GSK-3 β may have other binding partners as well.

β -Catenin has a consensus sequence phosphorylation site for GSK-3 β , and GSK-3 β 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-3 β consensus phosphorylation site of β -catenin prevent ubiquitination (Aberle *et al.*, 1997). Thus, it appears that GSK-3 β phosphorylates β -catenin and that the phosphorylation of β -catenin is essential for its degradation. Indeed, it has been reported

A

rat Axin	:	-----	
mouse Axin	1:	LGSGSRLREALAAAAAGACWGRAGANFQRLVVRVSSRFWRRSAACLAPPP	50
rat Axin	:	-----	
mouse Axin	51:	GHGSPSRRRRRDGGPPGPRRRGPPAEPLSAWASPGREP GPGPRLHSRR	100
rat Axin	1:	-----MQSPKMNVOEQGFPLDLGASFTEDAP	26
mouse Axin	101:	ALERLIPLGAVSTEVLGCSAHCSLMQSPKMNVOEQGFPLDLGASFTEDAP	150
rat Axin	27:	RPPVPGEEGELVSTDSRPVNHSFCSGKGTSIKSETSTATPRRSDLDLGYE	76
mouse Axin	151:	RPPVPGEEGELVSTDSRPVNHSFCSGKGTSIKSETSTATPRRSDLDLGYE	200
rat Axin	77:	PEGSASPTPPYLRAESLHSLDDQDGISLFRFLKQEGCADLLDFWFAC	126
mouse Axin	201:	PEGSASPTPPYLRAESLHSLDDQDGISLFRFLKQEGCADLLDFWFAC	250
rat Axin	127:	SGFRKLEPCDSNEEKRLKLARAIYRKYILDSNGIVSRQTKPATKSF IKDC	176
mouse Axin	251:	SGFRKLEPCDSNEEKRLKLARAIYRKYILDSNGIVSRQTKPATKSF IKDC	300
rat Axin	177:	VMKQOQIDPAMFDQAQTEIQSTMEENTYPSFLKSDIYLEYTRTGSES PKVC	226
mouse Axin	301:	VMKQOQIDPAMFDQAQTEIQSTMEENTYPSFLKSDIYLEYTRTGSES PKVC	350
rat Axin	227:	SDQSSSGSGTGKMGSGYLP TLNEDEEWKCDQDADEDDGRDVP	276
mouse Axin	351:	SDQSSSGSGTGKMGSGYLP TLNEDEEWKCDQDADEDDGRDVP	400
rat Axin	277:	LLETAAPRAPSSRRYNEGRELRYGSWREPVPYVNSGYALAPATSANDS	326
mouse Axin	401:	LLETAAPRAPSSRRYNEGRELRYGSWREPVPYVNSGYALAPATSANDS	450
rat Axin	327:	EQQSLSSDADTLSLTDSSVDGIPPYRIRKQHRREMQESIQVNGRVPLPHI	376
mouse Axin	451:	EQQSLSSDADTLSLTDSSVDGIPPYRIRKQHRREMQESI QVNGRVPLPHI	500
rat Axin	377:	PRTYRMPKEIRVEPQKFAEELIHRLEAVQRTREAEKLEERLKRVRMEEE	426
mouse Axin	501:	PRTYRMPKEIRVEPQKFAEELIHRLEAVQRTREAEKLEERLKRVRMEEE	550
rat Axin	427:	GEDGEMPSGPMASHKLPSVPAWHHFPPRYVDMGCSGLRDAHEENPESILD	476
mouse Axin	551:	GEDGEMPSGPMASHKLPSVPAWHHFPPRYVDMGCSGLRDAHEENPESILD	600
rat Axin	477:	EHVQVRMRTPGCQSPGPGHRSPDSGHVAKTAVLGGTASGHGKHAPKLGLK	526
mouse Axin	601:	EHVQVRMRTPGCQSPGPGHRSPDSGHVAKTAVLGGTASGHGKHV PKLGLK	650
rat Axin	527:	LDSAGLHHHRHVHHHVHNSARPKEQMEAEVARRVQSSFSWGPETHGHAK	576
mouse Axin	651:	LDTAGLHHHRHVHHHVHNSARPKEQMEAEVARRVQSSFSWGPETHGHAK	700
rat Axin	577:	PRSYSESTGTNFSAGDLAFGGKASAPSKRNTKKAESGKNASAEVPSTED	626
mouse Axin	701:	PRSYSEMAGTTLFSAGDLAFGGKASAPSKRNTKKAESGKNASAEVPSTED	750
rat Axin	627:	AEKNQKIMQWIIIEGEKEISRHRKAGHGSSGMRKQQAHESSRPLS IERPGA	676
mouse Axin	751:	AEKNQKIMQWIIIEGEKEISRHRKAGHGSSGMRKQQAHESSRPLS IERPGA	800
rat Axin	677:	VHPWVSAQLRNSVQPSHLFIQDPTMPPNPAPNPLTQLEEARRRLEEE EK R	726
mouse Axin	801:	VHPWVSAQLRNSVQPSHLFIQDPTMPPNPAPNPLTQLEEARRRLEEE EK R	850
rat Axin	727:	ANKLPSKQR-----TKSQR	740
mouse Axin	851:	ANKLPSKQRVYQAVMQRGRTCVRPACAPVLSVVPVAVSDLELSETE TKSQR	900
rat Axin	741:	KAGGGSAPPDCSIVVAYYFCGEPIPYRTLVRGRAVTLGQFKELLTKKGSY	790
mouse Axin	901:	KAGGGSAPPDCSIVVCYYFCGEPIPYRTLVRGRAVTLGQFKELLTKKGSY	950
rat Axin	791:	RYYFKKVSDEFDCGVVFEEVREDAI LPVFEEKIIGKVEKVD	832
mouse Axin	951:	RYYFKKVSDEFDCGVVFEEVREDEP LPVFEEKIIGKVEKVD	992

that in *Xenopus*, GSK-3 β phosphorylates β -catenin *in vitro* (Yost *et al.*, 1996). However, it has been shown that mammalian GSK-3 β 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-3 β on the degradation of β -catenin, we have tried to find a GSK-3 β -interacting protein using a yeast two-hybrid method. From a rat brain cDNA library, we have isolated a protein which interacts with GSK-3 β , 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-3 β and that it directly interacts with both GSK-3 β and β -catenin. Furthermore, we demonstrate that rAxin promotes GSK-3 β -dependent phosphorylation of β -catenin. These results suggest that Axin negatively regulates the Wnt signaling pathway by interacting with GSK-3 β and β -catenin and mediating the signal from GSK-3 β to β -catenin.

Results

Isolation of GSK-3 β -interacting protein

To identify proteins that physically interact with GSK-3 β , we conducted a rat brain cDNA library screening by the yeast two-hybrid method. From 3.7×10^6 initial transformants, four clones were found to confer both the His⁺ and LacZ⁺ phenotypes on L40 containing pBTM116HA/GSK-3 β . Among these, a 3.2 kb 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-3 β -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 M_r 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-3 β -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-3 β -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–

Fig. 1. Structure of rAxin. (A) Amino acid sequence of rAxin. Identical residues in rat and mouse Axin are denoted by a black background. The DDBJ/EMBL/GenBank Accession no. of rAxin is AF017756. (B) Schematic representation of rAxin. The RGS homologous domain, GSK-3 β -binding site, β -catenin-binding site and Dsh homologous domain are indicated. (C) Alignment of the amino acid sequences of rAxin and RGS4 or mouse DVL-1. Sequences of rAxin and RGS4 or DVL-1 are aligned using the GENETYX-MAC program. Amino acid identity is highlighted in black. Black dots under RGS4 indicate 11 amino acid residues that make direct contact with Gi1 α . (D) Northern blot analysis of rAxin. Total RNAs (20 μ g/lane) of various rat tissues were probed with cDNA encoding rAxin-(1–131). The positions of 28S and 18S rRNAs are indicated. The arrowhead indicates the positions of rAxin mRNA. The results shown are representative of two independent experiments.

Fig. 2. Interaction of rAxin with GSK-3 β in intact cells. (A) Expression in COS cells. The lysates (20 μ g of protein) expressing both Myc-rAxin and HA-GSK-3 β (lane 2), Myc-rAxin alone (lane 3) and HA-GSK-3 β alone (lane 4) were probed with the anti-Myc and GSK-3 β antibodies. The lysates of COS cells transfected with empty vectors were used as controls (lane 1). (B) Interaction of rAxin with GSK-3 β in COS cells. The same lysates (160 μ g of protein) of COS cells prepared in (A) were immunoprecipitated with the anti-Myc antibody (lanes 1–3) or the anti-HA antibody (lanes 4–6). The immunoprecipitates were probed with the anti-Myc and HA antibodies. (C) Interaction of rAxin with endogenous GSK-3 α and GSK-3 β . The lysates expressing Myc-rAxin (lanes 2 and 4) were immunoprecipitated with the anti-Myc antibody. The immunoprecipitates were probed with the anti-Myc, GSK-3 α and GSK-3 β antibodies. The lysates of COS cells transfected with empty vectors were used as controls (lanes 1 and 3). (D) Requirement for kinase activity of GSK-3 β for its interaction with rAxin. Myc-rAxin was co-expressed with wild-type HA-GSK-3 β (lane 1), HA-GSK-3 β ^{K85M} (lane 2) or HA-GSK-3 β ^{Y216F} (lane 3) in COS cells, and the lysates (160 μ g of protein) were immunoprecipitated with the anti-HA antibody. The precipitates were probed with the anti-Myc and HA antibodies. IP, immunoprecipitation; Ab, antibody; Ig, immunoglobulin; WT, wild-type HA-GSK-3 β ; 85M, HA-GSK-3 β ^{K85M}; 216F, HA-GSK-3 β ^{Y216F}. The large arrows, large arrowheads, small arrowheads and small arrow indicate the positions of Myc-rAxin, HA-GSK-3 β and its mutants, endogenous GSK-3 β and endogenous GSK-3 α , respectively. The results shown are representative of three independent experiments.

178 of RGS4, which has been identified as a GTPase-activating protein (GAP) for heterotrimeric GTP-binding protein (G protein) (Druey *et al.*, 1996) (Figure 1B and C). The C-terminal region, residues 757–820, shared 37% amino acid identity with residues 8–73 of the mouse Dsh homolog, DVL-1 (Sussman *et al.*, 1994) (Figure 1B and C). A single band of 4.8 kb *rAxin* mRNA was detected ubiquitously in those rat tissues which we examined (Figure 1D).

Interaction of rAxin with GSK-3 β

To examine whether rAxin interacts with GSK-3 β in intact cells, we co-expressed rAxin with GSK-3 β in COS cells (Figure 2A). rAxin and GSK-3 β were tagged with Myc and hemagglutinin 1 (HA) epitopes, respectively, at their N-termini. HA-GSK-3 β and endogenous GSK-3 β were detected by the anti-GSK-3 β antibody. When the lysates co-expressing Myc-rAxin with HA-GSK-3 β were immunoprecipitated with the anti-Myc antibody, HA-GSK-3 β 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-3 β immune complex (Figure 2B, lanes 4–6). Neither Myc-rAxin nor HA-GSK-3 β was immunoprecipitated from the lysates expressing

both proteins with non-immune immunoglobulin (data not shown). To show that Myc-rAxin interacts with endogenous GSK-3 α and GSK-3 β , the lysates expressing Myc-rAxin alone were immunoprecipitated with the anti-Myc antibody. Endogenous GSK-3 α and GSK-3 β were co-precipitated with Myc-rAxin (Figure 2C). These results suggest that rAxin forms a complex with GSK-3 α and GSK-3 β in intact cells. HA-GSK-3 β ^{K85M} and HA-GSK-3 β ^{Y216F}, in which ATP binding and tyrosine phosphorylation sites respectively are mutated, were expressed in COS cells. Neither HA-GSK-3 β ^{K85M} nor HA-GSK-3 β ^{Y216F} showed kinase activities for GSK peptide 1 (data not shown). Co-transfection of Myc-rAxin with these HA-GSK-3 β 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-3 β ^{K85M} or HA-GSK-3 β ^{Y216F} (Figure 2D). These results suggest that the kinase activity of GSK-3 β may be necessary for its complex formation with rAxin.

To determine which region of rAxin interacts with GSK-3 β , various deletion mutants of Myc-rAxin were expressed in COS cells (Figure 3A and B, upper panel). When the lysates expressing Myc-rAxin mutants were

Fig. 3. Interacting domain of rAxin with GSK-3 β . **(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-3 β . 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-3 β antibody (lower panel). IP, immunoprecipitation; Ab, antibody; Ig, immunoglobulin. The arrowhead indicates the position of endogenous GSK-3 β . The results shown are representative of three independent experiments.

immunoprecipitated with the anti-Myc antibody, endogenous GSK-3 β 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-3 β .

Phosphorylation of rAxin by GSK-3 β

To determine whether rAxin is a substrate for GSK-3 β , Myc-rAxin immunoprecipitated from the COS cell lysates was incubated with or without glutathione *S*-transferase (GST) fused to GSK-3 β (GST–GSK-3 β). rAxin was phosphorylated without GST–GSK-3 β , and this phosphorylation was enhanced by GST–GSK-3 β (Figure 4A). GST–GSK-3 β 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-3 β is co-precipitated with Myc-rAxin. Therefore, the phosphorylation of Myc-rAxin without GST–GSK-3 β might be due to the associated endogenous GSK-3 β . However, we cannot rule out the possibility that GSK-3 β 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-3 β .

To determine which regions of rAxin were phosphorylated by GSK-3 β , 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-3 β (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 phosphoryl-

ation site for GSK-3 β . Taken together with the observations in Figure 3, the physical interaction of rAxin with GSK-3 β may be required for the phosphorylation of rAxin.

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

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

To rule out the involvement of endogenous GSK-3 β 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-3 β phosphorylated MBP–rAxin-(298–506) in a time-dependent manner (Figures 4F and 5A). The K_m value for the phosphorylation of MBP–rAxin-(298–506) by GST–GSK-3 β was calculated to be 93 nM. The sequence S/TXXXS/T is known to be a consensus sequence for a GSK-3 β phosphorylation site (Plyte *et al.*, 1992). In residues 298–506 of rAxin, there are three possible phosphorylation sites: SANDSEQQS³³⁰, SDADTL³⁴¹SLT and SLTDS³⁴³. All serine and threonine residues of these possible phosphorylation sites were substituted by alanine, and the phosphorylation of these mutants by GST–GSK-3 β was examined. The phosphorylation levels of MBP–rAxin-(298–506, 322/326/330A), MBP–rAxin-(298–506, 333/337/341A) and MBP–rAxin-(298–506, 339/343A) by GST–GSK-3 β were reduced to 55–60% of wild-type (Figure 4F). These results indicate that rAxin is directly phosphorylated by GSK-3 β and that it has multiple phosphorylation sites for GSK-3 β .

Fig. 4. Phosphorylation of rAxin by GSK-3 β . **(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-3 β (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-3 β , respectively. **(B)** Time course. Myc-rAxin immunoprecipitated from the COS cell lysates was incubated with (●) or without (○) GST-GSK-3 β (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-3 β for 5 min. **(D)** Phosphorylation of rAxin mutants by GSK-3 β . 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-3 β (100 ng of protein) for 30 min. **(E)** Co-precipitation of endogenous Axin with GSK-3 β . The lysates (200 μg of protein) of COS cells (lanes 2 and 3) were immunoprecipitated with the anti-GSK-3 β 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-3 β . 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-3 β (100 ng of protein) for 15 min. As a control, MBP-rAxin-(298-506) was incubated without GST-GSK-3 β (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-3 β 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-3 β . Neither GST-GSK-3 β^{K85M} nor GST-GSK-3 β^{Y216F} 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-3 β mutants (Figure 5B). Since ATP was not used in

this experiment, it is suggested that the interaction of rAxin with GSK-3 β does not require the phosphorylation of rAxin but that the interaction may require the structure of GSK-3 β 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

Fig. 5. Kinetics of the phosphorylation of rAxin-(298-506) by GSK-3 β and the interaction of rAxin-(298-506) with GSK-3 β . (A) Direct phosphorylation of rAxin-(298-506) by GSK-3 β . MBP-rAxin-(298-506) (1 μg of protein) was incubated with (●) or without (○) GST-GSK-3 β (100 ng of protein) for the indicated time. (B) Direct binding of rAxin-(298-506) to GSK-3 β . GST-GSK-3 β (●), GST-GSK-3 β^{K85M} (○) and GST-GSK-3 β^{Y216F} (Δ) (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-3 β 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.

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

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 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) and 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.

Table I. Two-hybrid interactions between β -catenin and rAxin

	Growth on selective media (-His)		β -Galactosidase activity	
	pGAD	pGAD/ β -catenin	pGAD	pGAD/ β -catenin
pBTM116HA	-	-	-	-
pBTM116HA/rAxin	-	+	-	+

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 (Hulsken *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-3 β and β -catenin

From the results of Figures 3-6, we found that rAxin-(298-506) is phosphorylated by GSK-3 β and binds to both GSK-3 β and β -catenin. We therefore examined whether the phosphorylation of rAxin affects its interaction with GSK-3 β and β -catenin. When MBP-rAxin-(298-506) was incubated with GST-GSK-3 β in the presence or absence of ATP, GST-GSK-3 β 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-3 β and β -catenin.

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

The observations that rAxin directly binds to both GSK-3 β and β -catenin prompted us to examine whether these three proteins form a ternary complex. When Myc-rAxin and HA-GSK-3 β were co-expressed in COS cells and HA-GSK-3 β was immunoprecipitated with the anti-HA antibody, both Myc-rAxin and endogenous β -catenin were detected in the HA-GSK-3 β immune complex (Figure 8A, lanes 1 and 4). However, in the absence of Myc-rAxin, β -catenin was not detected in the HA-GSK-3 β immune complex (Figure 8A, lanes 2 and 5). Furthermore, Myc-rAxin-endogenous GSK-3 β complex was co-precipitated with GST- β -catenin, and inversely Myc-rAxin-endogenous β -catenin complex was co-precipitated with GST-GSK-3 β (Figure 8B). These results suggest that GSK-3 β ,

Fig. 7. Effect of the phosphorylation of rAxin on its interaction with GSK-3 β and β -catenin. (A) With GSK-3 β . MBP-rAxin-(298-506) (15 pmol) immobilized to amylose resin was incubated with the indicated concentrations of GST-GSK-3 β in the presence or absence of ATP for 30 min. MBP-rAxin-(298-506) was precipitated by centrifugation and the precipitates were probed with the anti-GST and MBP antibodies. (B) With β -catenin. MBP-rAxin-(298-506) (15 pmol) immobilized to amylose resin was incubated with GST-GSK-3 β (6 pmol) in the presence or absence of ATP for 30 min. The samples were washed to remove GST-GSK-3 β and ATP, then further incubated with the indicated concentrations of GST- β -catenin for 1 h. MBP-rAxin-(298-506) was precipitated by centrifugation and the precipitates were probed with the anti-GST and MBP antibodies. The positions of GST-GSK-3 β , GST- β -catenin and MBP-rAxin-(298-506) are indicated. The results shown are representative of three independent experiments.

rAxin and β -catenin form a ternary complex in intact cells and that GSK-3 β 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-3 β 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-3 β is distinct from the β -catenin-binding site.

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

It has been shown that β -catenin has a consensus sequence phosphorylation site for GSK-3 β 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-3 β regulates the stabilization of β -catenin. However, it has been reported that mammalian GSK-3 β does not

Fig. 8. Ternary complex formation of GSK-3 β , rAxin and β -catenin. (A) In COS cells. The lysates (20 μ g of protein) of COS cells expressing HA-GSK-3 β and Myc-rAxin (lanes 1 and 4) and HA-GSK-3 β alone (lanes 2 and 5) were probed with the anti-Myc, β -catenin and HA antibodies (lanes 1–3); the same lysates (200 μ g of protein) were immunoprecipitated with the anti-HA antibody and the immunoprecipitates were probed with the anti-Myc, β -catenin and HA antibodies (lanes 4–6). COS cells transfected with empty vectors were used as controls (lanes 3 and 6). (B) *In vitro*. After the lysates (200 μ g of protein) of COS cells expressing Myc-rAxin (lanes 2 and 4) or control cells (lanes 1 and 3) were incubated with GST- β -catenin (lanes 1 and 2) (18 pmol) or GST-GSK-3 β (lanes 3 and 4) (30 pmol) for 2 h, the samples were precipitated with glutathione-Sepharose 4B. The precipitates were probed with the anti-Myc, β -catenin and GSK-3 β antibodies. The arrows and large arrowheads indicate the positions of Myc-rAxin and β -catenin, respectively. The small arrowheads in (A) and (B) indicate the positions of HA-GSK-3 β and endogenous GSK-3 β , respectively. (C) Different binding sites of rAxin for GSK-3 β and β -catenin. After MBP-rAxin-(298–506) (lanes 1 and 5), MBP-rAxin-(353–437) (lanes 2 and 6), MBP-rAxin-(437–506) (lanes 3 and 7) and MBP (lanes 4 and 8) (30 pmol each) were incubated with GST-GSK-3 β (lanes 1–4) (30 pmol) or GST- β -catenin (lanes 5–8) (18 pmol) for 1 h, GST-GSK-3 β or GST- β -catenin was precipitated with glutathione-Sepharose 4B. The precipitates were probed with the anti-MBP antibody. The results shown are representative of three independent experiments.

phosphorylate β -catenin significantly *in vitro* (Rubinfeld *et al.*, 1996). Therefore, we examined whether GSK-3 β 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-3 β 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-3 β 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-3 β -dependent phosphorylation of β -catenin. Furthermore, GST- β -catenin-(1–423) but not GST- β -catenin-(423–781) was phosphorylated by GST-GSK-3 β 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-3 β . Since MBP-rAxin-(298–506) does not increase the phosphorylation of GSK peptide 1 by GST-GSK-3 β (data not shown), it is unlikely that rAxin activates GSK-3 β kinase activity. Taken together, these results suggest that rAxin plays a role in placing β -catenin in the vicinity of GSK-3 β , thereby enhancing the phosphorylation of β -catenin by GSK-3 β .

Discussion

We have isolated a protein which interacts with GSK-3 β 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 Siamese-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-3 β 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-3 β , GSK-3 β^{K85M} and GSK-3 β^{Y216F} . It has been reported that insect cell-expressed GSK-3 β^{Y216F} showed 10% activity of the wild-type (Hughes *et al.*, 1993), but our results have shown that neither HA-GSK-3 β^{Y216F} in COS cells nor bacterially expressed GST-GSK-3 β^{Y216F} exhibit kinase activities. Although we do not know the reasons why GSK-3 β^{Y216F} kinase activities are different

Fig. 9. Phosphorylation of β -catenin by GSK-3 β in the presence of rAxin. (A and D) GST- β -catenin (4 μ g of protein) was incubated with the indicated amounts of GST-GSK-3 β in the presence (+, $\bullet$) or absence (-, $\circ$) of MBP-rAxin-(298-506) (200 ng of protein) for 30 min. (B and E) GST- β -catenin (2 μ g of protein) was incubated with GST-GSK-3 β (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-3 β (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-3 β , respectively. The results shown are representative of four independent experiments.

among expression systems, our results suggest that GSK-3 β without kinase activity does not have the ability to bind to rAxin. It is known that recombinant GSK-3 β has a specific activity less than that of mammalian expressed protein (Hughes *et al.*, 1993). Our *in vitro* experiments have shown that GST-GSK-3 β binds to MBP-rAxin-(298-506). These results suggest that the interaction of GSK-3 β with rAxin requires its kinase activity and that even a low specific kinase activity is sufficient for the interaction. Alternatively, the interaction of GSK-3 β with rAxin may require the structure of GSK-3 β 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-3 β in a binding conformation. We have also demonstrated that rAxin is phosphorylated by GST-GSK-3 β and that rAxin-(298-506) contains at least one phosphorylation site for GST-

GSK-3 β . Since we have used immunoprecipitated rAxin as a GST-GSK-3 β 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-3 β and phosphorylate rAxin. However, MBP-rAxin-(298-506) is phosphorylated by GST-GSK-3 β , and mutations in the consensus sequence phosphorylation sites for GSK-3 reduce the phosphorylation of MBP-rAxin-(298-506) by GST-GSK-3 β . Therefore, it is likely that GSK-3 β directly phosphorylates rAxin and that SANDSEQQS³³⁰, SDADTL³⁴¹SLT³⁴¹ and SLTD³⁴³ are the phosphorylation sites for GSK-3 β . 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-3 β 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-3 β does not require prior phosphorylation.

Our results have shown that rAxin interacts not only with GSK-3 β 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-3 β increases the binding of APC to β -catenin (Rubinfeld *et al.*, 1996). However, the phosphorylation of rAxin by GSK-3 β does not affect its binding to GSK-3 β and β -catenin. Therefore, we do not know the physiological significance of the phosphorylation of rAxin by GSK-3 β .

We have demonstrated that rAxin, GSK-3 β and β -catenin form a ternary complex in COS cells. Consistent with these observations, GSK-3 β and β -catenin bind to separate sites on rAxin, and these sites are adjacent. Furthermore, we have found that rAxin promotes GSK-3 β -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-3 β 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-3 β 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-3 β -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 G α and stimulates GTP hydrolysis of G α . 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 G α GAP and that Axin thereby inhibits the Wnt signaling pathway. The three-dimensional structure of a stable complex of RGS4 and Gi α 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 Gi1 α 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 Gi2 α nor binds to the GTP-bound form of Gi2 α (unpublished data). So far, all of the RGS proteins currently characterized bind to members of the Gi α class of G protein α subunits, including Go α , Gz α and Gq α . Therefore, the RGS domain of rAxin may regulate the G protein activity of members other than the Gi α class. Alternatively, it may have an additional activity of transmitting the signal by interacting with other protein(s). It has been shown that Δ 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 Δ RGS (Zeng *et al.*, 1997). These results indicate that Δ 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. Our observation that the RGS domain is distinct from the GSK-3 β - 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-3 β and β -catenin promotes GSK-3 β -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-3 β , pBSSK/ β -catenin, pGAD/ β -catenin, a λ ZAP rat brain cDNA library, pEF-BOS, pGEX-KG, a peptide substrate of GSK-3 (GSK peptide 1), the anti-HA antibody

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.Tsukita (Kyoto University, Kyoto, Japan), S.Kuroda and K.Kaibuchi (Nara Institute 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.Tamanoi (University of California, Los Angeles, CA), C.W.Turck (University of California, San Francisco, CA) (Murai *et al.*, 1996), Q.Hu (Chiron Corp., CA) and M.Nakata (Sumitomo Electric Industries, Yokohama, Japan), respectively. The anti-Myc antibody was prepared from 9E10 cells. GST-GSK-3 β 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-3 β and β -catenin antibodies were purchased from Transduction Laboratories (Lexington, KY), and the anti-GSK-3 α antibody was from UBI (Lake Placid, NY). [α -³²P]dCTP and [γ -³²P]ATP were obtained from Amersham Inc. (Buckinghamshire, UK). Other materials were from commercial sources.

Plasmid construction

To construct pBTM116HA/GSK-3 β , pBSSK/GSK-3 β was digested with *Bcl*I and *Eco*RI, and blunted with Klenow fragment. The 1.3 kb fragment was inserted into pBTM116HA which was digested with *Bam*HI and blunted with Klenow fragment. To construct pBSKS/rAxin, the 0.2 kb fragment encoding rAxin-(1–70) with a *Sma*I site at the 5' end was synthesized by PCR. This fragment was digested with *Sma*I and *Bgl*III and inserted into the *Sma*I- and *Bgl*III-cut pBSKS containing the 3.2 kb fragment encoding rAxin prepared from the library in pGAD10. pBSKS/rAxin was digested with *Sma*I and *Eco*RV, and the 3.2 kb fragment encoding rAxin was inserted into pMAL-c2 which was digested with *Xba*I and blunted with Klenow fragment to construct pMAL-c2/rAxin. To construct pBTM116HA/rAxin, pMAL-c2/rAxin was digested with *Eco*RI and *Sal*I, and the 3.2 kb fragment encoding rAxin was inserted into the *Eco*RI- and *Sal*I-cut pBTM116HA. The 1.3 kb fragments encoding GSK-3 β ^{K85M} and GSK-3 β ^{Y216F} with *Xba*I and *Sma*I sites synthesized by PCR were inserted into the *Xba*I- and *Sma*I-cut pCGN to construct pCGN/GSK-3 β ^{K85M} and pCGN/GSK-3 β ^{Y216F}. pCGN/GSK-3 β and pGEX-2T/GSK-3 β were constructed as described (Murai *et al.*, 1996). To construct pGEX-2T/GSK-3 β ^{K85M} and pGEX-2T/GSK-3 β ^{Y216F}, the 1.3 kb fragments encoding GSK-3 β ^{K85M} and GSK-3 β ^{Y216F} with *Eco*RV and *Eco*RI sites synthesized by PCR were digested with *Eco*RV and *Eco*RI and inserted into the *Sma*I- and *Eco*RI-cut pGEX-2T. To construct pEF-BOS-Myc, the fragment encoding the Myc epitope synthesized by PCR was inserted into pEF-BOS which was digested with *Xba*I and blunted with Klenow fragment. To construct pEF-BOS-Myc/rAxin, pBSKS/rAxin was digested with *Sma*I and *Eco*RV, and the 3.2 kb fragment encoding rAxin was inserted into pEF-BOS-Myc which was digested with *Xba*I and blunted with Klenow fragment. pBSKS/rAxin was digested with *Sac*I, and the 1.7 kb fragment encoding rAxin-(298–832) was inserted into the *Sac*I-cut pUC19 to construct pUC19/rAxin-(298–832). pUC19/rAxin-(298–832) was digested with *Eco*RI and *Sma*I and blunted with Klenow fragment, and the 1.7 kb fragment encoding rAxin-(298–832) was inserted into pBJ-Myc which was digested with *Bam*HI and blunted with Klenow fragment to construct pBJ-Myc/rAxin-(298–832). To construct pBSKS/rAxin-(508–832), pBSKS/rAxin was digested with *Pst*I and *Eco*RV and blunted with T4 DNA polymerase. The 1.7 kb fragment encoding rAxin-(508–832) was inserted into the *Sma*I-cut pBSKS. After *Spe*I linker was inserted into the *Eco*RV-cut pBSKS/rAxin-(508–832), pBSKS/rAxin-(508–832) was digested with *Spe*I, and the 1.7 kb fragment encoding rAxin-(508–832) was inserted into the *Xba*I-cut pEF-BOS-Myc to construct pEF-BOS-Myc/rAxin-(508–832). To construct pBSKS/rAxin-(713–832), pBSKS/rAxin was digested with *Pvu*II and *Eco*RV, and the 1.0 kb fragment encoding rAxin-(713–832) was inserted into the *Sma*I-cut pBSKS. To construct pBJ-Myc/rAxin-(713–832), pBSKS/rAxin-(713–832) was digested with *Xba*I and *Eco*RV, and the 1.0 kb fragment was inserted into pBJ-Myc which was digested with *Bam*HI, blunted with Klenow fragment and digested with *Xba*I. To construct pBJ-Myc/rAxin-(1–229), pBSKS/rAxin was digested with *Sac*I and *Sma*I and blunted with T4 DNA polymerase. The 0.69 kb fragment was inserted into pBJ-Myc, which was digested with *Xba*I and blunted with Klenow fragment. To construct pEF-BOS-Myc/rAxin-(1–713), the 0.94 kb fragment encoding rAxin-(401–713) with *Xba*I sites was synthesized by PCR, digested with *Xba*I and inserted into the *Xba*I-cut pEF-BOS-Myc/rAxin. To construct pBJ-Myc/rAxin-(298–713), Myc epitope-tagged rAxin-(298–713) with *Eco*RI sites synthesized by PCR was digested with *Eco*RI and inserted into *Eco*RI-cut pBJ-1. To construct pEF-BOS-Myc/rAxin-(298–506), pBJ-Myc/rAxin-

(298–832) was digested with *Eco*RI and *Pst*I, blunted with T4 DNA polymerase, and the 0.68 kb fragment was inserted into pEF-BOS which was digested with *Xba*I and blunted with Klenow fragment. To construct pMAL-c2/Myc-rAxin-(298–506), pBJ-Myc/rAxin-(298–832) was digested with *Eco*RI and *Pst*I, and the 0.68 kb fragment was inserted into the *Eco*RI- and *Pst*I-cut pMAL-c2. To construct pUC19/rAxin-(298–506, 322/326/330A), the 0.62 kb fragment encoding rAxin-(298–506) in which Ser322, Ser326 and Ser330 were substituted by Ala, was synthesized by PCR, digested with *Sac*I and *Bgl*III and inserted into the *Sac*I- and *Bam*HI-cut pUC19. To construct pMAL-c2/rAxin-(298–506, 322/326/330A), pUC19/rAxin-(298–506, 322/326/330A) was digested with *Eco*RI and *Hind*III, and the fragment was inserted into the *Eco*RI- and *Hind*III-cut pMAL-c2. To construct pMAL-c2/rAxin-(298–506, 333/337/341A) and pMAL-c2/rAxin-(298–506, 339/343A), the same procedures as the construction of pMAL-c2/rAxin-(298–506, 322/326/330A) were performed. To construct pEF-BOS-Myc/rAxin-(353–506), pBSKS/rAxin was digested with *Bam*HI and *Pst*I and blunted with T4 DNA polymerase. The 0.46 kb fragment encoding rAxin-(353–506) was inserted into pEF-BOS-Myc which was digested with *Xba*I and blunted with Klenow fragment. pEF-BOS-Myc/rAxin-(353–506) was digested with *Bam*HI and *Nco*I and blunted with Klenow fragment. The 0.25 kb fragment encoding rAxin-(353–437) was inserted into pMAL-c2 which was digested with *Xba*I and *Pst*I and blunted with T4 DNA polymerase to construct pMAL-c2/rAxin-(353–437). To construct pMAL-c2/rAxin-(437–506), pBJ-Myc/rAxin-(298–713) was digested with *Nco*I and blunted with Klenow fragment, then digested with *Pst*I. The 0.21 kb fragment encoding rAxin-(437–506) was inserted into pMAL-c2 which was digested with *Xba*I and blunted with Klenow fragment, then digested with *Pst*I. To construct pGEX-2T/ β -catenin, pBSSK/ β -catenin was digested with *Xho*I and blunted with Klenow fragment, and the 2.7 kb fragment was inserted into the *Sma*I-cut pGEX-2T. To construct pGEX-2T/ β -catenin-(1–423), pBSSK/ β -catenin was digested with *Bam*HI and *Eco*RI, and the 1.3 kb fragment encoding β -catenin-(1–423) was inserted into the *Bam*HI- and *Eco*RI-cut pGEX-2T. To construct pGEX-KG/ β -catenin-(423–781), pBSKS/ β -catenin was digested with *Eco*RI and *Xho*I, and the 1.4 kb fragment encoding β -catenin-(423–781) was inserted into the *Eco*RI- and *Xho*I-cut pGEX-KG. To construct pGEX-2T/ β -catenin-(1–131), pGEX-2T/ β -catenin-(132–423) and pGEX-2T/ β -catenin-(175–423), the fragments encoding β -catenin-(1–131), β -catenin-(132–423) and β -catenin-(175–423) with *Bam*HI and *Eco*RI sites were synthesized by PCR, digested with *Bam*HI and *Eco*RI, and inserted into the *Bam*HI- and *Eco*RI-cut pGEX-2T.

Yeast two-hybrid screening

Yeast strain L40 (*MATa trp1 leu2 his3 ade2 LYS2::lexA-HIS3 URA3::lexA-lacZ*) was used as a host for the two-hybrid screening (Vojtek *et al.*, 1993). A strain L40 carrying pBTM116HA/GSK-3 β was transformed with a rat brain cDNA library constructed in pGAD10. pBTM116HA/GSK-3 β directs the expression of a fusion between the DNA-binding domain of LexA and the entire GSK-3 β from an *ADH* promoter. Approximately 3.7×10^6 transformants were screened for the growth on SD plate media lacking tryptophan, leucine and histidine as evidenced by transactivation of a *LexA-HIS3* reporter gene and histidine prototrophy. His⁺ colonies were scored for β -galactosidase activity. Plasmids harboring cDNAs were recovered from positive colonies and the nucleotide sequences of plasmid DNAs which conferred the LacZ⁺ phenotype on L40 containing pBTM116HA/GSK-3 β were determined.

Molecular cloning of rAxin

To obtain full-length cDNA of GSK-3 β -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 λ ZAP rat 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-3 β , GSK-3 α and β -catenin with rAxin in COS cells

COS cells (10 cm diameter dish) transfected with pCGN-, pBJ- 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-3 β and its mutants were tagged with HA epitope at their N-termini. The lysates (50–800 μ g of protein) were immunoprecipitated with the anti-Myc or HA antibody, then the precipitates were probed with the anti-Myc, HA, GSK-3 β , GSK-3 α and

β -catenin antibodies (Hinoi *et al.*, 1996; Kikuchi and Williams, 1996; Murai *et al.*, 1997).

Interaction of GSK-3 β and β -catenin with rAxin in vitro

To determine the K_d value of rAxin binding to GSK-3 β , GST–GSK-3 β (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 μ l reaction mixture [20 mM Tris–HCl pH 7.5 and 1 mM dithiothreitol (DTT)]. GST–GSK-3 β 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 GSK-3 β with rAxin was examined, the wild-type and various mutants of GST–GSK-3 β or GST– β -catenin were incubated with various mutants of MBP–rAxin in 40 μ l reaction mixture or with the lysates (200 μ g of protein) of COS cells expressing Myc–rAxin for 2 h at 4°C. GST– β -catenin, GST–GSK-3 β and their mutants were precipitated with glutathione–Sepharose 4B, then the precipitates were probed with the anti-MBP, Myc, β -catenin and GSK-3 β antibodies.

GSK-3 β kinase assay

The kinase activities of GSK-3 β and its mutants for GSK peptide 1 were measured as described (Murai *et al.*, 1996). The lysates (150 μ g of protein) of COS cells (6 cm diameter dish) expressing wild-type and mutated HA–GSK-3 β were immunoprecipitated with the anti-HA antibody. The precipitates were incubated with 50 μ M GSK peptide 1 in 30 μ l of kinase reaction mixture [50 mM Tris–HCl pH 7.5, 10 mM MgCl₂, 1 mM DTT and 50 μ M [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 P81) and washed with phosphoric acid (Murai *et al.*, 1996). When the immunoprecipitated Myc–rAxin was used as a substrate, the lysates (50–800 μ g 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–GSK-3 β (100 ng of protein) in 30 μ l of the kinase reaction mixture for 30 min at 30°C. When the phosphorylation of rAxin-(298–506) and β -catenin by GST–GSK-3 β 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 GSK-3 β , MBP–rAxin-(298–506) (15 pmol) immobilized to amylose resin was incubated with various concentrations of GST–GSK-3 β with or without 50 μ M ATP in 30 μ l 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 amylose resin was incubated with GST–GSK-3 β (6 pmol) with or without 50 μ M ATP in 30 μ l 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–GSK-3 β and ATP. After the precipitates were incubated with various amounts of GST– β -catenin in 30 μ l 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 amylose 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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