Interaction among GSK-3, GBP, Axin, and APC in Xenopus Axis Specification

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Abstract. Glycogen synthase kinase 3 (GSK-3) is a constitutively active kinase that negatively regulates its substrates, one of which is β-catenin, a downstream effector of the Wnt signaling pathway that is required for dorsal-ventral axis specification in the Xenopus embryo. GSK-3 activity is regulated through the opposing activities of multiple proteins. Axin, GSK-3, and β-catenin form a complex that promotes the GSK-3-mediated phosphorylation and subsequent degradation of β-catenin. Adenomatous polyposis coli (APC) joins the complex and downregulates β-catenin in mammalian cells, but its role in Xenopus is less clear. In contrast, GBP, which is required for axis formation in Xenopus, binds and inhibits GSK-3. We show here that GSK-3 binding protein (GBP) inhibits GSK-3, in part, by preventing Axin from binding GSK-3. Similarly, we present evidence that a dominant-negative GSK-3 mutant, which causes the same effects as GBP, keeps endogenous GSK-3 from binding to Axin. We show that GBP also functions by preventing the GSK-3-mediated phosphorylation of a protein substrate without eliminating its catalytic activity. Finally, we show that the previously demonstrated axis-inducing property of overexpressed APC is attributable to its ability to stabilize cytoplasmic β-catenin levels, demonstrating that APC is impinging upon the canonical Wnt pathway in this model system. These results contribute to our growing understanding of how GSK-3 regulation in the early embryo leads to regional differences in β-catenin levels and establishment of the dorsal axis.

Key words: Wnt pathway • dorsal/ventral • β-catenin

Introduction

The Wnt family of secreted glycoproteins are important mediators of a variety of developmental processes across animal phyla, and also have roles in cell proliferation and oncogenesis. From genetic data obtained in Drosophila and biochemical and cell biological data in Xenopus and mammalian cell culture, a great deal has been learned about this developmentally crucial pathway (for reviews see Dale, 1998; Wodarz and Nusse, 1998). Wnts signal through receptors of the frizzled class (Bhanot et al., 1996; Yang-Snyder et al., 1996; He et al., 1997), leading to the hyperphosphorylation of Dishevelled (Yanagawa et al., 1995), a protein of unknown function. Activation of Dishevelled leads to the inhibition of the serine/threonine kinase glycogen synthase kinase 3 (GSK-3) (Noordermeer et al., 1994; Siegfried et al., 1994; Wagner et al., 1997), which, in the absence of Wnt signaling, binds Axin and phosphorylates β-catenin (Yost et al., 1996; Hart et al., 1998; Ikeda et al., 1998; Kishida et al., 1998; Nakamura et al., 1998; Sakanaka et al., 1998), targeting it for ubiquitination and subsequent degradation by the proteosome pathway (Aberle et al., 1997; Orford et al., 1997). Therefore, Wnt signaling results in the stabilization of β-catenin by inhibiting GSK-3. Cytoplasmic β-catenin associates with HMG box class transcription factors of the Lef/Tcf families (Behrens et al., 1996; Miller and Moon, 1996; Molenaar et al., 1996) to activate the transcription of target genes (Brannon et al., 1997; Laurent et al., 1997; Mckendry et al., 1997).

A large body of evidence has implicated the Wnt pathway in the establishment of the early dorsal signaling center in Xenopus (for reviews see Harland and Gerhart, 1995).
In response to sperm entry, a microtubule array is established that causes a rotation of a thin layer of cortical cytoplasm towards the side opposite sperm entry (Elionson and Rowning, 1988). Cortical rotation leads to the movement of a transplantable dorsalizing activity from the vegetal pole of the egg to the future dorsal side of the embryo (Fujisue et al., 1993; Kikkawa et al., 1996; Sakai, 1996; Rowning et al., 1997). Positive effectors of the Wnt pathway, when overexpressed ventrally, mimic this endogenous dorsalizing activity (Mooon and Kimelman, 1998). However, the role of more upstream members of the pathway, Wnt itself and Dishevelled, is still unclear. Dominant-negative versions of these proteins do not affect axis formation (Hoppeler et al., 1996; Sokol, 1996), but it may not be possible to introduce these constructs early enough to affect endogenous axis formation. Two recent findings leave open the possibility that these upstream components of the pathway may play a role. First, Dishevelled has been shown recently to be enriched dorsally in one-cell embryos, and ectopic GFP-tagged Dishevelled is transported along the microtubule array during cortical rotation (Millier et al., 1999). Second, a maternal Wnt, Wnt-11, has been shown recently to be asymmetrically distributed at the protein level as a result of asymmetric polyadenylation, which is dependent on cortical rotation (Schroeder et al., 1999).

Numerous studies indicate that the dorsal determinant functions to inhibit GSK-3 activity. A kinase dead GSK-3 acts as a dominant-negative, duplicating the axis when expressed ventrally (Dominguez et al., 1995; He et al., 1995; Pierce and Kimelman, 1995), and a β-catenin mutant that lacks the GSK-3 phosphorylation sites necessary for its degradation is a more potent axis inducer than the wild-type protein (Yost et al., 1996). β-Catenin is required for axis formation (Heasman et al., 1994) and is enriched dorsally by the two-cell stage in a manner dependent on cortical rotation (Larabel et al., 1997). The dorsal accumulation of β-catenin activates transcription of dorsal-specific genes such as siamois (Branon et al., 1997) and Xnr-3 (McEndry et al., 1997). Finally, the embryonic cytoplasm containing the dorsalizing activity can cause nuclear accumulation of β-catenin and induce expression of siamois and Xnr3 (Darras et al., 1997; Mairikawa et al., 1997).

With β-catenin established as the direct regulator of gene transcription downstream of Wnt signaling, and GSK-3 established as the direct regulator of cytoplasmic β-catenin levels, attention has shifted to the question of how GSK-3 itself is regulated in the early embryo. Two novel families of GSK-3 binding proteins (GBP) have been identified, and both clearly have been shown to regulate GSK-3 function, although in opposite ways. The first of these families of GSK-3 binding proteins includes Xenopus GBP and the mammalian FRATs (Jonkers et al., 1997; Yost et al., 1998). GBP is required for the formation of the endogenous Xenopus axis, and both GBP and FRAT2 have axis-inducing activity when ectopically expressed in Xenopus (Yost et al., 1998). Ectopic GBP stabilizes β-catenin levels in Xenopus (Yost et al., 1998), and FRAT1 elevates the level of cytosolic β-catenin in NIH 3T3 cells (Yuan et al., 1999). GBP inhibits the ability of GSK-3 to phosphorylate a protein substrate, tau, in an in vivo assay, suggesting that GBP inhibits the kinase function of GSK-3 (Yost et al., 1998). The presence of mammalian homologues, and the fact that FRAT1 was cloned as a cooperating oncogene which confers a selective advantage to Myc and Pim1-expressing tumors (Jonkers et al., 1997), suggest that this family of GSK-3 inhibitors is important in processes besides Xenopus axis formation. At present, GBP is the most upstream component shown to be required for specification of the endogenous dorsal axis, though whether it plays a role in all Wnt-mediated signaling events is an open question. It is possible that GBP activates a unique maternal Wnt-related intracellular pathway, independent of Wnt ligand.

The second of these GSK-3 binding protein families includes Axin (Zeng et al., 1997) and the related proteins Axil (Yamamoto et al., 1998) and Conductin (Behrens et al., 1998). Axin functions as a scaffolding protein that directly binds both GSK-3 and its substrate β-catenin (Hart et al., 1998; Ikeda et al., 1998; Kishida et al., 1998; Nakamura et al., 1998; Sakanaka et al., 1998) and greatly enhances GSK-3's ability to phosphorylate β-catenin (Hart et al., 1998; Ikeda et al., 1998), leading to its degradation (Abriel et al., 1997; Hart et al., 1998). Axin mutations in the mouse embryo lead to axis duplication, and overexpression of Axin on the dorsal side of the Xenopus embryo abolishes the endogenous axis (Zeng et al., 1997). In Drosophila, D-Axin is required for the negative regulation of Wg signaling (Hamada et al., 1999; Willert et al., 1999). d-axin mutant clones contain elevated levels of the Drosophila homologue of β-catenin, Arm (Hamada et al., 1999), and D-Axin interacts with Arm and Zeste-white 3 (Willert et al., 1999). Axin is a maternal protein, present throughout development (Zeng et al., 1997; Hamada et al., 1999; Hedgepeth et al., 1999) and found throughout adult mouse tissues (Zeng et al., 1997). The ubiquitous expression of Axin, and its presence in both vertebrates and invertebrates, suggests that it plays a role in a broad range of GSK-3-regulated processes.

Axin functions as a part of a multiprotein complex that also includes the APC tumor suppressor protein (for reviews see Polakis, 1997; Bienz, 1999). APC directly binds Axin and β-catenin to a protein complex that includes GSK-3 (Rubinfeld et al., 1996; Hart et al., 1998; Kishida et al., 1998). Cell culture experiments have implicated APC in the downregulation of β-catenin (Munemitsu et al., 1995; Hayashi et al., 1997; Hart et al., 1998), although studies in Xenopus have suggested that APC has an alternative role, activating dorsal axis formation in a pathway requiring β-catenin but independent of β-catenin stabilization (Velenickx et al., 1997). Thus, a picture has begun to emerge wherein multiple proteins with opposing effects converge on GSK-3 to regulate its activity and, therefore, cytoplasmic β-catenin levels. The next question is how are these various inputs on GSK-3 activity integrated to regulate the level of β-catenin available for transcription of target genes? We sought to build on our previous work showing that GBP inhibits GSK-3 by determining how this inhibition occurs, and specifically, how GBP opposes the kinase function of GSK-3 (Yost et al., 1998). The presence of mammalian homologues, and the fact that FRAT1 was cloned as a cooperating oncogene which confers a selective advantage to Myc and Pim1-expressing tumors (Jonkers et al., 1997), suggest that this family of GSK-3 inhibitors is important in processes besides Xenopus axis formation. At present, GBP is the most upstream component shown to be required for specification of the endogenous dorsal axis, though whether it plays a role in all Wnt-mediated signaling events is an open question. It is possible that GBP activates a unique maternal Wnt-related intracellular pathway, independent of Wnt ligand.
in Xenopus axis formation by determining the effects of overexpressed A PC on β-catenin levels in the embryo.

We show here, both in vivo and in vitro, that GSK-3 cannot bind GBP and A xin simultaneously, leading to the model that GBP functions in part to prevent GSK-3 binding to the A xin/APC/β-catenin complex. We show that dnX-gsk-3 binds A xin in vivo, and propose that it functions to induce an ectopic axis in a manner analogous to GBP by keeping endogenous X enopus GSK-3 (X gsk-3) from binding to A xin. A dditionally, we demonstrate that GBP inhibits X gsk-3-mediated phosphorylation of protein substrates without eliminating the kinase activity of X gsk-3. Finally, we show that the A PC constructs that duplicate the axis in Xenopus also stabilize β-catenin, providing an explanation for this surprising result. These results provide a framework for understanding how positive and negative regulators affect GSK-3 activity and, subsequently, β-catenin levels.

Materials and Methods

RNA Expression Constructs

The FLAG-epitope-tagged GBP mutant that does not bind X gsk-3 (BP339), myc-epitope-tagged X gsk-3 (X G134), and FLAG-epitope-tagged wild-type GBP (BP20) have been described previously (Yost et al., 1998). A xin-myc (Zeng et al., 1997), β-catenin-myc (XBC40; Yost et al., 1996), and XAPCFL, X A PC1, and X A PC4 (Viemnickx et al., 1997) also have been described. X gsk-3-FLAG (X G140) was cloned by selecting the X gsk-3 fragment from XG134 into CS2 + FLAG. GBP-myc (BP25) was constructed by cloning the GBP fragment from BP20 into CS2 + MT (Turner and Weintraub, 1994), and the volume was increased to 1.5 ml with binding buffer. Proteins were nuted at 4°C for 1 h. 50 μl of prereduced galtathione resin was added to each reaction and proteins were nuted an additional hour at 4°C. The resin was spun down for 1 min at 1,000 rpm and washed four times in 1 ml PBS, 0.5% NP-40. Sample buffer was added directly to the beads, and proteins were run on a 15% polyacrylamide gel. Alternatively, 4 μg of MBP or MBP-Axin was incubated with 10 μg BSA and 7 μl of [35S]J GSK-3 translation mix, in a final volume of 1 ml binding buffer. Proteins were collected with 50 μl of prereduced amylase resin.

Results

GSK-3 Cannot Bind GBP and A xin Simultaneously

The observations that A xin binds GSK-3 and β-catenin to promote the phosphorylation of β-catenin by GSK-3 (Hart et al., 1998; Ikeda et al., 1998; Yamamoto et al., 1998), and that GBP binds and inhibits GSK-3 (Yost et al., 1998), led us to ask if GBP might function by preventing GSK-3 from binding A xin. Immunoprecipitations and in vitro pull-down assays were performed to address this question. In the first set of experiments, we asked if the presence of an excess of GBP could reduce the amount of A xin immunoprecipitated with X gsk-3, when the levels of the latter two were kept constant. RNA constructs encoding FLAG-epitope-tagged X gsk-3 (X G3-FLAG) and myc-epitope-tagged GBP (GBP-myc) and A xin (A xin-myc) (Zeng et al., 1997) were injected into the animal region of two- to eight-cell stage Xenopus embryos. Embryos were lyzed after 4–5 h, and immunocomplexes were precipitated with anti-FLAG antibodies. The immunoprecipitates were analyzed on a blot with both anti-FLAG and anti-myc antibodies. In preliminary experiments, injection of a combination of 50 pg of X gsk-3 RNA and 1 ng of A xin RNA was shown to result in the production of an excess of A xin protein relative to X gsk-3 (data not shown). Coinjection of increasing amounts of GBP RNA with this mixture of X gsk-3 and A xin RNA resulted in a decrease in A xin immunoprecipitated with X gsk-3 (Fig. 1 a, lanes 2–6), though both were produced at the same level in all samples. To demonstrate that the reduction in the amount of A xin bound to X gsk-3 is specifically
Figure 1. (a) GBP blocks GSK-3 binding to Axin in vivo. Embryos were injected at the two to eight-cell stage in the animal pole with 50 pg X gsk-3-FLAG and 1 ng A Axin-myc along with increasing doses of GBP-myc, as indicated. After 4-5 h, proteins were isolated by immunoprecipitation with anti-FLAG antibody and detected by Western blotting (left panel). An aliquot of each sample taken before immunoprecipitation is shown in the right panel (Total Lysates); the level of Xgsk-3 produced is not detectable in these samples (1 embryo). A portion of the total lysate shown in lane 6 was precipitated in the absence of anti-FLAG antibody as a negative control (lane 7). Lane numbers in the right panel refer to the same injections as shown above the corresponding lane numbers in the left panel. (b) Prevention of GBP binding to Xgsk-3-FLAG by GBP is dependent on GBP/GSK-3 binding (Fig. 1 b). These results indicate that GBP can prevent the association of Axin and Xgsk-3, either by displacing Axin from Xgsk-3, or by preventing the formation of Axin-Xgsk-3 complexes.

In a complementary set of experiments, we asked if GBP could join a GSK-3-Axin complex. For these experiments, the GSK-3 binding region of rat Axin, RAxin (198-306), which binds GSK-3 as well as full-length Axin (Ikedo et al., 1998), was purified as a maltose binding protein fusion (MBP-Axin) and GSK-3 was purified as a glutathione-S-transferase fusion (GST-GSK-3β). GBP was synthesized, sulfur-35-labeled in vitro, and was added to mixtures of GST-GSK-3β and increasing amounts of MBP-Axin or MBP. After an incubation, GST-GSK-3β was collected on glutathione-conjugated Sepharose beads, and the levels of [35S]GBP associated with GST-GSK-3β were determined by SDS-PAGE and autoradiography. Whereas [35S]GBP did not significantly bind the glutathione resin (Fig. 2 a, lane 2) or GST (data not shown), it effectively bound GST-GSK-3β (Fig. 2 a, lane 3). Inclusion of increasing amounts of MBP-Axin with GST-GSK-3β resulted in less [35S]GBP binding to GST-GSK-3β (Fig. 2 a, lanes 4-7). At a 50-fold molar excess of MBP-Axin to GST-GSK-3β, binding of [35S]GBP was completely eliminated compared with the background (Fig. 2 a, compare lanes 6 and 2). This effect is specifically dependent on MBP-Axin binding to GST-GSK-3β since substituting even a 1,000-fold molar excess of maltose binding protein (MBP) does not significantly affect [35S]GBP binding (Fig. 2 a, lane 11).

While these results were consistent with the hypothesis that GSK-3 can be bound to either GBP or Axin, it was also possible that MBP-Axin binds GBP and this interaction prevents GBP from interacting with GSK-3. To test this, we examined the ability of MBP-Axin to pull down [35S]GBP. As shown in Fig. 2 b, whereas MBP-Axin effect-
tively binds [35S]GSK-3 (lane 6), it does not interact with [35S]GBP (Fig. 2b, lane 4). Moreover, M B-P-A xin does not associate with [35S]GSK-3 when [35S]GBP is present (Fig. 2b, lane 5), supporting the idea that the three proteins do not form a ternary complex.

GBP Does Not Inhibit the Ability of Xgsk-3 to Phosphorylate a Peptide Substrate

The demonstration that GBP and A xin cannot bind GSK-3 simultaneously suggests that GBP might inhibit GSK-3 by removing it from the A xin complex or by preventing GSK-3 from associating with A xin. Since we previously demonstrated that GBP inhibits the in vivo phosphorylation of tau (Yost et al., 1998), a protein not thought to be involved in Wnt signaling, we wanted to examine whether GBP might also be able to inhibit GSK-3 by binding and inactivating the catalytic site. We used a modification of a published assay for GSK-3 activity that measures the ability of GSK-3 to phosphorylate the peptide substrate P-CREB, which contains a GSK-3 consensus phosphorylation site, in comparison to the negative control peptide, CREB (Wang et al., 1994). Embryos were injected with RNA encoding Xgsk-3-myc together with control RNA or GBP-FLAG RNA. After 3 h, proteins were extracted and immunoprecipitated. Anti-FLAG antibodies were used to isolate Xgsk-3 bound to GBP when both were injected; anti-myc antibodies were used to isolate Xgsk-3 when it was injected with a control RNA; and un.injected embryos were immunoprecipitated with both antibodies to measure background. The immunoprecipitates were incubated with γ-[32P]ATP in kinase buffer containing P-CREB or CREB, and the incorporated radioactivity was quantified. Western blotting showed that GBP and Xgsk-3 were both expressed and efficiently immunoprecipitated, and remained associated throughout the assay (data not shown). As shown in Fig. 3, GBP does not affect the ability of Xgsk-3 to phosphorylate this peptide substrate. This shows that GBP can inhibit GSK-3 in a way that does not inactivate its catalytic activity.

The Dominant-negative Xgsk-3 Binds Axin

It has been shown in a number of studies, including our own, that mutation of a conserved lysine in the ATP binding region of GSK-3 results in a kinase-deficient mutant that acts as a dominant-negative mutant (dnX gsk-3) in Xenopus (Dominguez et al., 1995; He et al., 1995; Pierce and Kimelman, 1995). The demonstration that A xin binds GSK-3 and promotes the phosphorylation of β-catenin suggests that the dnX gsk-3 might function by binding A xin and keeping it from binding endogenous X gsk-3. However, studies in a mammalian system have shown that kinase dead GSK-3 does not bind A xin (Ikeda et al., 1998), indicating that the kinase dead dnX gsk-3 might stabilize β-catenin by an alternative mechanism. To investigate this issue, we compared X gsk-3 and dnX gsk-3 binding to A xin in coimmunoprecipitation experiments. A xin-myc was coexpressed with either X gsk-3-FLAG or dnX gsk-3-FLAG in Xenopus embryos, and expressed proteins were immunoprecipitated with anti-FLAG antibodies. Immune complexes were analyzed by Western blotting with anti-myc and anti-FLAG antibodies. As expected, A xin is immunoprecipitated by X gsk-3 (Fig. 4, lane 7). In addition, A xin is immunoprecipitated equally well by dnX gsk-3 (Fig. 4, lane 8). Thus, unlike in the mammalian system, the kinase dead mutant of X gsk-3 binds A xin, suggesting that it stabilizes β-catenin either by displacing endogenous X gsk-3 from the A xin complex, or by preventing association of A xin and endogenous X gsk-3.

Truncated APC Stabilizes β-Catenin

Various lines of evidence have implicated APC in the downregulation of β-catenin. In the colon cancer cell line SW 480, for example, a mutation that truncates the Wnt pathway, could be involved in promoting tumor cell growth and metastasis.
20-amino acid repeat region leads to the accumulation of β-catenin (for review see Polakis, 1997). When full-length APC, or deletion constructs containing the central 20-amino acid repeat region, are introduced into these cells, β-catenin levels are reduced (Munemitsu et al., 1995). Given these results, APC would be predicted to have a ventralizing activity in *Xenopus*. However, ectopic expression of APC, or the deletion mutants that lower β-catenin levels in cells, on the ventral side of *Xenopus* embryos resulted in a duplicated dorsal axis and the induction of a β-catenin–dependent gene, *siamois* (Vleminckx et al., 1997). These effects would be expected if β-catenin was stabilized by the ectopic APC, but no stabilization was detected. Cytoplasmic β-catenin was required for this effect, however, because coexpression of C-cadherin, which sequesters β-catenin to the plasma membrane, abolished axis duplication by ectopic APC. Therefore, it was proposed that in *Xenopus*, APC functions as a positive regulator of dorsal axis formation in a manner not requiring β-catenin stabilization, but dependent upon cytoplasmic β-catenin (Vleminckx et al., 1997).

Since we were interested in understanding this potentially novel means of β-catenin regulation by APC, we repeated the ectopic expression experiments with APC. Like Vleminckx et al. (1997), we observed that ectopic full-length *Xenopus* APC (XAPCFL) has axis-inducing activity (Fig. 5 b). Whereas the previous study measured the ability of XAPCFL to induce a dorsal axis when ectopically expressed on the ventral side of the embryo, we measured the ability of XAPCFL to induce an axis in embryos whose endogenous axis has been ablated by UV light. Whereas the latter assay (the UV rescue assay) is more easily quantified than secondary axis formation, the two assays measure the same process.

When we examined the expression of ectopic XAPCFL, using the myc-epitope tag located at the NH2 terminus, we noticed that the APC protein migrated as a series of forms that were less than the expected molecular mass of 320 kD (Fig. 5 c). While the exact pattern of these smaller molecular mass forms detected varied between experiments, the truncated forms were always observed to be the major products produced. Since the myc-epitope tag is located at
the NH₂ terminus of the APC constructs, the smaller forms we observe are due to COOH-terminal truncations. XAPC1 and XAPC4, which also induce an ectopic axis (Fig. 5 b and Vleminkx et al., 1997), migrated at their expected molecular mass (Fig. 5 c).

We next wished to determine if the observed truncated protein products we observed when we injected XAPCFL might be generated during processing of the embryos for immunoprecipitation or if they were present in the intact cells. Lysate from uninjected embryos was immunoprecipitated with antibody to APC (Rubinfeld et al., 1993) in parallel with lysate from embryos injected with XAPCFL, which was immunoprecipitated with antibody to the myc epitope. A single band of endogenous APC was detected in the uninjected sample (Fig. 5 d, lane 1), whereas injected siblings produced a variety of faster migrating species in addition to full-length myc-tagged APC (Fig. 5 d, lane 3). Blotting the XAPCFL-injected sample with the anti-APC2 antibody shows that this antibody is capable of recognizing the larger of the truncated products (Fig. 5 d, lane 5). Because this antibody was generated against only the central third of APC, it would not be expected to recognize truncations that contain only the NH₂-terminal third or less of APC. Because endogenous APC appeared degraded, the lysis and immunoprecipitation protocols are not responsible for the observed truncated XAPCFL products. Importantly, this indicates that these truncated products are actually present in the embryo before lysis.

Next, we wished to reexamine the results obtained by Vleminkx et al. (1997) in a β-catenin stabilization experiment, in which an assay we developed (Yost et al., 1996) was used to claim that β-catenin levels were unchanged by injection of full-length XAPC RNA into Xenopus embryos. Low levels of ectopically expressed myc-tagged β-catenin are used to measure the rate of β-catenin degradation in this assay, which is very sensitive to the dose of injected β-catenin-myc (Yost et al., 1996). At the dose of β-catenin-myc used by Vleminkx et al. (1997) (1 ng), we were concerned that it was not possible to measure the degradation of the ectopically expressed β-catenin since it overwhelms the endogenous degradation machinery. To test this, embryos were injected with a range of doses of β-catenin-myc from 10 pg to 500 pg, with or without 1 ng of GBP-myc to stabilize β-catenin levels, and with a control RNA (GBP) to equalize the mass of RNA injected for each treatment (Fig. 6 a). When 50 pg or less of β-catenin-myc was used, there was an increase in the levels of β-catenin protein when GBP-myc was coinjected with β-catenin-myc. Above this dose, however, β-catenin levels were identical in the presence and absence of GBP. Therefore, the previous measurement of β-catenin stability by Vleminkx et al. (1997) would not have given a meaningful result. To reexamine whether ectopic XAPCFL stabilizes β-catenin, embryos were injected with two doses of XAPCFL RNA along with 50 pg β-catenin-myc RNA. The high dose of XAPCFL caused significant axis rescue in UV-irradiated embryos, whereas the low dose did not (not shown). Correspondingly, the high dose of XAPCFL stabilized β-catenin, whereas the low dose did not (Fig. 6 b). Because the major product produced from injection of XAPCFL RNA was similar in size to the NH₂-terminal fragment XAPC1 (Fig. 5 c), and because XAPC4 contains the β-catenin binding domain, we next asked if these two constructs could rescue the axis and stabilize β-catenin. Both XAPC1 and XAPC4 caused significant axis rescue in UV-irradiated embryos (Fig. 5 b). The β-catenin stabilization experiment was performed using a dose of RNA that was at least as high as that required to give good axis rescue. In this experiment, β-catenin tagged with an HA epitope was used so that the myc-epitope-tagged XAPC constructs would not interfere with the detection of the ectopic β-catenin. Both XAPC1 and XAPC4 were found to enhance the levels of β-catenin (Fig. 6 c). Because axis rescue and β-catenin stabilization were observed with the deletion constructs, and because a variety of truncated products accumulate in the intact embryo when XAPCFL is overexpressed, it is likely that the effects seen with XAPCFL are due to the production of truncated products.
**Discussion**

While the most upstream components of the Wnt signaling pathway, Wnt and Disherel, are sufficient to induce an ectopic axis when overexpressed in *Xenopus* (McMahon and Moon, 1989; Smith and Harland, 1991; Sokol et al., 1991; Rothbacher et al., 1995; Sokol et al., 1995), neither has been shown to be required for formation of the endogenous axis. This suggests the interesting possibility that the downstream components of the pathway, including the serine/threonine kinase GSK-3, are regulated in a unique way in the early embryo, independent of Wnt ligand. The regulation of GSK-3 has been shown to be critical for proper dorsal-ventral axis specification in *Xenopus* (Domínguez et al., 1995; H et al., 1995; Pierce and Kimelman, 1995). GSK-3 functions as part of a multiprotein complex in which Axin directly binds GSK-3, β-catenin, and APC (H et al., 1998; Ikeda et al., 1998; Itoh et al., 1998; Nakamura et al., 1998; Sakanaka et al., 1998; Yamasato et al., 1998), and promotes the phosphorylation of β-catenin by GSK-3 (H et al., 1998; Ikeda et al., 1998; Yamasato et al., 1998). We previously demonstrated that GBP inhibits the Xgsk-3-mediated phosphorylation of an in vivo protein substrate, tau, in a manner dependent on GBP binding to Xgsk-3 (Yost et al., 1998). Therefore, Axin and GBP both bind to GSK-3, but have opposite effects on its activity. Based on the data implicating Axin in the positive regulation of GSK-3, we hypothesized that GBP might function to prevent GSK-3 from binding Axin. Using both in vivo and in vitro approaches, we show here that GBP and Axin do not simultaneously bind to GSK-3.

In embryos, when a limiting amount of Xgsk-3 was coexpressed with an excess of Axin, addition of GBP reduced the amount of Xgsk-3 bound to Axin. In vitro, when GBP was limiting, increasing amounts of Axin prevented GBP from binding to GSK-3. Axin did not bind GBP in vitro, either directly or in the presence of GSK-3. Moreover, when Axin, Xgsk-3, and GBP were coexpressed in embryos at comparable levels, immunoprecipitation of GBP brought down Xgsk-3 but not Axin, demonstrating that these proteins cannot form a trimolecular complex (data not shown). Importantly, it also has been observed recently in mammalian cells that the GBP homologue, FRAT1, reduces the amount of Axin that can be communoprecipitated with GSK-3 (Yuan et al., 1999), demonstrating that this mechanism of GSK-3 regulation is conserved among vertebrates. Taken together, the in vivo and in vitro data support a model in which GBP binds to Xgsk-3 and wild-type Xgsk-3 bind Axin equally well in vivo. This is consistent with a model in which overexpressed kinase-deficient Xgsk-3 acts as a dominant-negative either by displacing endogenous Xgsk-3 already bound to Axin or by preventing endogenous Xgsk-3 from binding Axin as new complexes form. In either case, endogenous Xgsk-3 is prevented from phosphorylating β-catenin by the dominant-negative Xgsk-3 because it is prevented from associating with Axin. In mammalian cells, the lack of a dominant-negative activity for kinase-deficient GSK-3 can be attributed to its inability to bind Axin. It is unclear why a more severe mutation in a mammalian GSK-3 than that of Ikeda et al. (1998) apparently retains its ability to bind Axin in vitro (Sakanaka et al., 1998). However, it was not addressed whether this mutant acted as a dominant-negative.

**GBP Does Not Inhibit GSK-3 Kinase Activity**

Since the inhibition of tau phosphorylation requires GBP binding to Xgsk-3 (Yost et al., 1998), we hypothesized that GBP could bind to GSK-3 in a manner that inactivates its catalytic cleft. This mechanism has been shown for the interaction between Cdk2, which shares a high degree of sequence homology with GSK-3, and the Kip/Cip family of CDK inhibitors, which includes p21Cip1/WAF1, p27Kip1, and p57Kip2. When p27Kip1 binds to Cdk2, it causes large structural changes in the NH₂ terminus and catalytic cleft, which eliminates ATP binding and kinase activity (Russo et al., 1996). Using a peptide phosphorylation assay, we find that Xgsk-3 kinase activity is not inhibited by GBP binding. Additionally, FRAT1 does not affect the ability of GSK-3 to phosphorylate a peptide substrate (Yuan et al., 1999). Taken together, the results from the tau assay and the peptide assay suggest that GBP binds to GSK-3 in a manner that does not inhibit the catalytic activity of the active site, unlike the case for p27Kip1 binding to Cdk2. GBP might sterically block access of protein substrates to the active site of GSK-3, and we are currently attempting to map the residues of Xgsk-3 that are important for GBP binding to Xgsk-3 to determine if GBP might bind in the region of GSK-3’s active site. These results also show that this widely used assay of GSK-3 activity in some important cases may not accurately reflect the extent of GSK-3 functional inhibition.

**The Dominant-negative Xgsk-3 Binds Axin**

Mutation of a lysine residue, conserved in all kinases, in the ATP binding domain of GSK-3 creates a kinase-deficient protein that acts as a dominant-negative mutant in *Xenopus*. Overexpression of this mutant on the ventral side of embryos results in the formation of a second body axis (Domínguez et al., 1995; H et al., 1995; Pierce and Kimelman, 1995) by locally preventing the degradation of β-catenin (Yost et al., 1996; Larabell et al., 1997). It was previously assumed that dnXgsk-3 functions by competing with endogenous Xgsk-3 for substrates or regulatory molecules. The demonstration that Axin binds GSK-3 and β-catenin and promotes GSK-3 phosphorylation of β-catenin suggested that Axin might be the target of the dnXgsk-3. However, Ikeda et al. (1998) showed that a kinase-deficient mammalian GSK-3 analogous to ours does not bind Axin in cell culture, and other workers have found that similar GSK-3 mutants do not act as dominant-negatives in the Wnt pathway in mammalian cells (Woodgett, J., personal communication). A different GSK-3 kinase mutant, GSK-3ΔY > F also communoprecipitates less Axin than wild-type GSK-3 in mammalian cells (Yuan et al., 1999). Furthermore, A kt, which inhibits GSK-3 by phosphorylating it, reduces the amount of Axin communoprecipitated by GSK-3 (Yuan et al., 1999). However, we show here that the dominant-negative Xgsk-3 and wild-type Xgsk-3 bind Axin equally well in vivo. This is consistent with a model in which overexpressed kinase-deficient Xgsk-3 acts as a dominant-negative either by displacing endogenous Xgsk-3 already bound to Axin or by preventing endogenous Xgsk-3 from binding Axin as new complexes form. In either case, endogenous Xgsk-3 is prevented from phosphorylating β-catenin by the dominant-negative Xgsk-3 because it is prevented from associating with Axin. In mammalian cells, the lack of a dominant-negative activity for kinase-deficient GSK-3 can be attributed to its inability to bind Axin. It is unclear why a more severe mutation in a mammalian GSK-3 than that of Ikeda et al. (1998) apparently retains its ability to bind Axin in vitro (Sakanaka et al., 1998). However, it was not addressed whether this mutant acted as a dominant-negative.
**Overexpressed APC Induces Axis Formation by Stabilizing β-Catenin**

APC appears to be an important regulator of β-catenin levels and, as such, has been implicated in cancer progression and in development (for reviews see Polakis, 1997; Bienz, 1999). Evidence from tissue culture suggests that APC functions in conjunction with GSK-3 and Axin to downregulate β-catenin levels, and that GSK-3 phosphor-

ulates both APC and β-catenin as a prerequisite for β-catenin degradation (Rubinfield et al., 1996). Data obtained from a study overexpressing APC in X enopus, however, suggested that the role of APC in frog embryos might be different. Vleminckx et al. (1997) showed that APC could induce an ectopic axis and induce expression of siamois, apparently without stabilizing β-catenin, though cytoplasmic β-catenin was required. The authors concluded that X enopus APC has a signaling role independent of β-catenin regulation.

Studies in C. elegans have shown that Wnt signaling is also involved in establishing cell polarity in the EMS cell division (Rocheleau et al., 1997; Thorpe et al., 1997). Downstream components of the Wnt pathway, however, including POP-1 (TCF), WRM-1 (β-catenin), and APR-1 (APC), may be used differently in worms than in other organisms (for review see Han, 1997). For example, WRM-1 is required for excluding POP-1 from the nuclei of the E cell, allowing that cell to develop as an endoderm (Lin et al., 1998). In vertebrates, in contrast, β-catenin interacts with Tcf-1 in the nucleus to activate transcription. Furthermore, RNAi loss-of-function experiments have shown that apr-1(rnaI) and wrm-1(rnaI) C. elegans embryos have similar defects in E blastoderm development, suggesting that a PR-1 positively regulates WRM-1 (Rocheleau et al., 1997).

Our results support a model in which APC acts to down-regulate β-catenin in X enopus. We found that injection of RNA encoding full-length APC (XAPCFL) in X enopus results in the accumulation of a number of COOH terminally truncated products, presumably because of either incomplete translation or proteolytic cleavage in the embryo. While we (Fig. 5 d) and Vleminckx et al. (1997) did detect a high molecular mass APC product from injected RNA, both studies also observed the presence of truncated species. We also found that a level of XAPCFL, XAPC1, or XAPC4 RNA that rescues axis formation in UV-irradiated embryos also stabilizes β-catenin levels. Thus, we conclude that the APC products expressed from the injected RNA induce an ectopic axis by increasing β-catenin levels, as is observed when Wnt (Larabell et al., 1997), β-catenin (Funayama et al., 1995; Y ost et al., 1996), dnX gsk-3 (Y ost et al., 1996), or GBP (Y ost et al., 1998) are overexpressed in X enopus embryos. No alternate or parallel pathway needs be invoked to explain the axis-inducing effect of ectopically expressed APC in X enopus.

Because the various deletion constructs cause axis formation through β-catenin stabilization, we propose that the effects of ectopic XAPCFL are mediated not by the full-length protein, but by the abundant truncated products. We suggest that the ability of ectopic APC to induce an axis in X enopus is a dominant-negative effect in which the truncated APC products displace the endogenous APC from its normal partners. XAPC4, for example, contains two of the three A xin binding domains identified in A PC (Behrens et al., 1998; Hart et al., 1998), and is likely to displace endogenous APC from the A xin-X gsk-3 complex, preventing it from participating in the degradation of β-catenin. Supporting this interpretation, it has been shown that an A PC fragment that binds Conductin stabilizes β-catenin in Neuro2A cells and prevents APC-induced degradation of β-catenin in SW480 cells (Behrens et al., 1998). However, a fragment of human APC similar to XAPC4 reduces cytoplasmic β-catenin levels in SW480 cells (Munemitsu et al., 1995). The different effects of this central part of APC on β-catenin stability may be accounted for by differences in the systems examined. For example, a dominant-negative effect may depend on full-length A PC, present in X enopus but not in SW480 cells, or that more protein may be produced by overexpression in X enopus than transfection of mammalian cells. Unlike Vleminckx et al. (1997), we also observed axis rescue by XAPC1, which is the most NH2-terminal fragment and lacks the identified A xin binding domains (Fig. 5 a). This fragment contains an NH2-terminal oligomerization domain (Su et al., 1993), which could bind the endogenous A PC and block its function, and also a domain of A rm repeats, which might be expected to mediate an interaction between A PC and an unidentified partner.

**Figure 7. Model for GSK-3 regulation in the early X enopus embryo.** (a) On the ventral side of the embryo, GSK-3 is part of a functional degradation complex that includes APC and A xin. When in this complex, GSK-3 phosphorylates β-catenin, targeting it for degradation via the proteosome pathway. Under these conditions, dorsal genes are repressed. (b) On the dorsal side of the embryo, GSK-3 is excluded from the A xin-APC complex by GBP. In addition, GBP may prevent GSK-3 from phosphorylating its normal substrates by blocking access to the active site. β-Catenin accumulates and activates the transcription of dorsal genes. (c) The kinase-deficient dnxgsk-3 functions by binding A xin, thus, keeping endogenous X gsk-3 from the degradation complex. A s in the situation with GBP, β-catenin accumu-

lates and activates the transcription of dorsal genes.
**Model for GSK-3 Dorsal–Ventral Regulation in Xenopus**

The coordinated regulation of GSK-3 activity by both positive and negative regulators is critical for a wide range of downstream effects, from insulin regulation to developmental processes (for review see Y ost et al., 1997). The experiments presented here advance our understanding of GSK-3 regulation and allow us to propose the following model for X gsk-3 regulation in the fertilized Xenopus egg. On the ventral side, β-catenin levels are kept low by X gsk-3-dependent phosphorylation in a complex including A xin and A PC (Fig. 7 a). Phosphorylation of A xin and A PC by X gsk-3 may be required to assemble this complex. On the dorsal side, GBP inhibits phosphorylation of β-catenin either by displacing X gsk-3 from the A xin/A PC/X gsk-3 complex or by prebinding GSK-3 and preventing its association with the A xin–A PC complex. GBP binding to X gsk-3 may block access of protein substrates to the active site (Fig. 7 b). GBP might also prevent X gsk-3 from phosphorylating A xin and A PC, and thereby further inhibit complex formation. The dominant-negative X gsk-3 acts in a manner analogous to GBP since it either displaces wild-type X gsk-3 from the complex, or binds endogenous X gsk-3 and prevents its binding to A xin, thus, inhibiting β-catenin degradation (Fig. 7 c).

The complete chain of molecular events linking fertilization and the dorsal enrichment of β-catenin has yet to be determined, but this study brings us closer to understanding the interactions of key players. It will ultimately be determined, but this study brings us closer to understanding the interactions of key players. It will ultimately be determined, but this study brings us closer to understanding the interactions of key players.

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