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)[1] (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 inhibition of 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,

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[1]*Abbreviations used in this paper:* APC, adenomatous polyposis coli; dnXgsk-3, dominant-negative Xgsk-3; GSK, glycogen synthase kinase 3; GST, glutathione-S-transferase; MBP, maltose binding protein; Xgsk-3, *Xenopus* GSK-3.

1997; Heasman, 1997; Moon and Kimelman, 1998). 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 (Elinson 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 (Moon 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 (Hoppler 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 (Miller et al., 1999). Second, a maternal Wnt, Wnt-11, has been shown recently to be asymetrically 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 (Larabell et al., 1997). The dorsal accumulation of β-catenin activates transcription of dorsal-specific genes such as *siamois* (Brannon et al., 1997) and *Xnr-3* (McKendry 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; Marikawa 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 NIH3T3 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 (Aberle 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 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 in 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 (Vleminckx 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 activity of Axin. To gain further insight into the relationship between Axin and GSK-3, we investigated the mechanism of action of the dominant-negative *Xenopus* GSK-3 (dnXgsk-3). Finally, we sought to clarify the role of APC

in *Xenopus* axis formation by determining the effects of overexpressed APC on β-catenin levels in the embryo.

We show here, both in vivo and in vitro, that GSK-3 cannot bind GBP and Axin simultaneously, leading to the model that GBP functions in part to prevent GSK-3 binding to the Axin/APC/β-catenin complex. We show that dnXgsk-3 binds Axin in vivo, and propose that it functions to induce an ectopic axis in a manner analogous to GBP by keeping endogenous *Xenopus* GSK-3 (Xgsk-3) from binding to Axin. Additionally, we demonstrate that GBP inhibits Xgsk-3–mediated phosphorylation of protein substrates without eliminating the kinase activity of Xgsk-3. Finally, we show that the APC 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 Xgsk-3 (BP139), myc-epitope–tagged Xgsk-3 (XG134), and FLAG-epitope–tagged wild-type GBP (BP20) have been described previously (Yost et al., 1998). Axin-myc (Zeng et al., 1997), β-catenin-myc (XBC40; Yost et al., 1996), and XAPCFL, XAPC1, and XAPC4 (Vleminckx et al., 1997) also have been described. Xgsk-3-FLAG (XG140) was constructed by cloning the Xgsk-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 GBP-HA was constructed by cloning this fragment into CS2 + HA, a derivative of CS2+ (Turner and Weintraub, 1994) containing an HA epitope (details available upon request). BP139-HA was constructed by cloning the mutant GBP from BP139 into CS2 + HA, and an HA-epitope–tagged β-catenin was constructed by subcloning β-catenin from XBC40 into CS2 + HA to generate XBC-HA. A FLAG-epitope–tagged dominant-negative Xgsk-3 (XG210) was constructed as follows: the Xgsk-3–containing BamHI fragment from XG21 (Pierce and Kimelman, 1995) was cloned into CS2+ to make XG114. The HindIII-EcoRV fragment of XG140 was replaced with the same fragment from XG114 to generate XG210.

Embryos and Microinjection

Embryos were obtained as previously described (Newport and Kirschner, 1982). UV ventralization of embryos was performed as described (Gerhart et al., 1989). Embryos were microinjected (Moon and Christian, 1989) with RNA synthesized from CS2+ (Turner and Weintraub, 1994) -derived constructs linearized with NotI or Asp718 using the mMessage mMachine kit (Ambion) according to the manufacturer's instructions.

Production of Proteins

rAxin (298-506) (Ikeda et al., 1998) was produced as an MBP fusion in *Escherichia coli* (MBP-Axin). Recombinant MBP and MBP-Axin proteins were purified over amylose resin (New England Biolabs) and eluted with maltose according to the manufacturer's directions. GSK-3 was produced as a glutathione-S-transferase fusion in *E. coli* (GST-GSK-3) (Ikeda et al., 1998), purified over glutathione resin (Pharmacia Biotech), and eluted with 20 mM glutathione. Kinase activity was confirmed using the peptide phosphorylation assay as described (Wang et al., 1994). ^{35}S-labeled proteins were produced in Promega TNT coupled reticulocyte lysate systems according to the manufacturer's directions.

Immunoprecipitation, Western Blotting, and Pull-down Assays

Immunoprecipitations and Western blotting were performed as described (Yost et al., 1998), except for experiments involving detection of endogenous APC. In this case, embryo lysates, prepared as described (Yost et al., 1998), were electrophoresed on 4–12% gradient gels (Novex) and transferred to PVDF in Tris-glycine buffer lacking SDS and methanol. Endogenous APC was immunoprecipitated and detected with anti-APC2 antibody (Rubinfeld et al., 1993; provided by P. Polakis). Anti-MBP antibody was purchased from New England Biolabs, anti-GST antibody was purchased from Amersham Pharmacia Biotech, and anti-HA antibody was purchased from Santa Cruz Biotechnology. Extinction coefficients for MBP, MBP-Axin, and GST-GSK-3 were calculated using ExPASy ProtParam and protein concentrations were determined by UV absorbance at 280 nm. 4 μg of GST-GSK-3 was incubated at room temperature with the specified molar excess concentrations of MBP and MBP-Axin for 10 min with 15 μg RNaseH in binding buffer (20 mM Tris, pH 7.4, 200 mM NaCl). After this short incubation, 15 μg BSA and 9 μl of the [^{35}S]GBP translation mix were added and the volume was increased to 1,500 μl with binding buffer. Proteins were nutated at 4°C for 1 h. 50 μl of prerinsed glutathione resin was added to each reaction and proteins were nutated 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 the [^{35}S]GBP ± [^{35}S]GSK-3 translation mix, in a final volume of 1 ml binding buffer. Proteins were collected with 50 μl of prerinsed amylose resin.

CREB Peptide Kinase Assay

Immunoprecipitates were washed as usual in NP-40 lysis buffer and were washed additionally with 50 mM Tris, pH 8.0, plus 1 M NaCl, and finally in 50 mM Tris, pH 8.0. Kinase reactions were carried out essentially as described (Wang et al., 1994) in a 30-μl reaction volume for 20 min at 30°C. Levels of Xgsk-3-myc immunoprecipitated alone or with GBP were quantified using densitometry and phosphorus-32 incorporation was normalized to Xgsk-3 protein levels. CREB peptide and the prephosphorylated p-CREB peptide were synthesized by Genosys.

Results

GSK-3 Cannot Bind GBP and Axin Simultaneously

The observations that Axin 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 Axin. Coimmunoprecipitations 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 Axin coimmunoprecipitated with Xgsk-3, when the levels of the latter two were kept constant. RNA constructs encoding FLAG-epitope–tagged Xgsk-3 (*Xgsk-3-FLAG*) and myc-epitope–tagged GBP (*GBP-myc*) and Axin (*Axin-myc*) (Zeng et al., 1997) were injected into the animal region of two- to eight-cell stage *Xenopus* embryos. Embryos were lysed 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 *Xgsk-3* RNA and 1 ng of *Axin* RNA was shown to result in the production of an excess of Axin protein relative to Xgsk-3 (data not shown). Coinjection of increasing amounts of *GBP* RNA with this mixture of *Xgsk-3* and *Axin* RNAs resulted in a decrease of Axin coimmunoprecipitated with Xgsk-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 Axin bound to Xgsk-3 is specifically

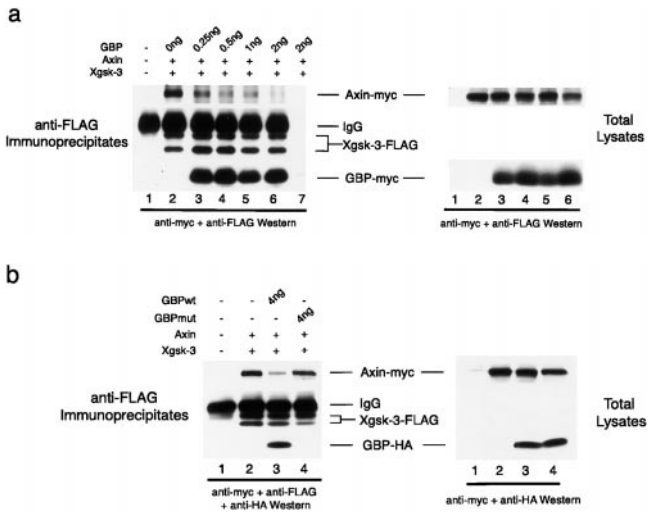

Figure 1. (a) GBP blocks GSK-3 binding to Axin in vivo. Embryos were injected at the two to eight-cell stage in the animal pole with 50 pg *Xgsk-3-FLAG* and 1 ng *Axin-myc* along with increasing doses of *GBP-myc*, as indicated. After 4–5 h, proteins were isolated by immunoprecipitation with anti-FLAG 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 GSK-3–Axin binding by GBP is dependent on GBP/GSK-3 binding. Embryos were injected with *Axin-myc* and *Xgsk-3-FLAG* as above along with 4 ng HA-tagged wild-type (GBPwt) or the HA-tagged point mutant that does not bind GSK-3 (GBPmut). Immunoprecipitations were performed as above. No GBP is present in the anti-FLAG immunoprecipitates in lane 4 since this is the GSK-3 binding mutant of GBP. Lane numbers in the right panel refer to the same injections as shown above the corresponding lane numbers in the left panel.

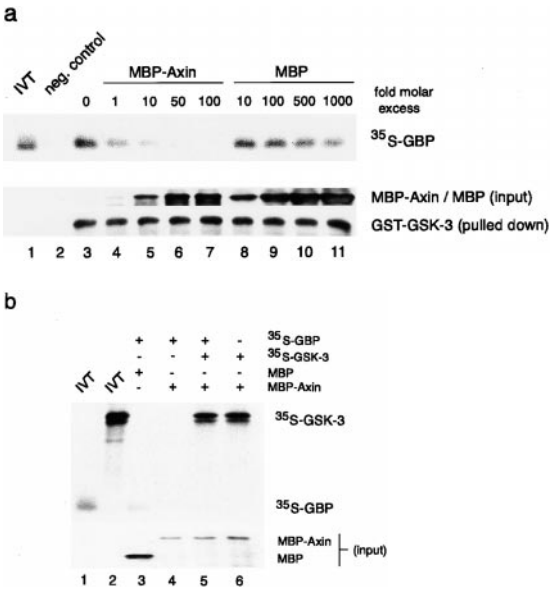

Figure 2. (a) GBP and Axin do not bind GSK-3 at the same time. Bacterially produced GST-GSK-3β and MBP-Axin or MBP were incubated with [35S]methionine-labeled GBP. MBP-Axin or MBP was present in excess over GST-GSK-3β as indicated. Protein complexes were precipitated with glutathione-Sepharose beads and [35S]GBP was detected by SDS-PAGE and autoradiography. MBP-Axin/MBP levels were detected by SDS-PAGE and anti-MBP Western blotting of 0.33% input. IVT, an aliquot of the in vitro translation reaction; 11% of binding reaction input. Neg. control, [35S]GBP incubated with glutathione-Sepharose beads. The level of GST-GSK-3 pulled down in each sample was detected by anti-GST Western blotting. (b) GBP does not bind MBP-Axin directly or through an interaction with GSK-3. Bacterially produced MBP-Axin was incubated with [35S]methionine-labeled in vitro translated GBP in the presence or absence of [35S]methionine-labeled Xgsk-3. Protein complexes were precipitated with amylose resin, and GBP and Xgsk-3 were detected by SDS-PAGE and autoradiography. IVT, an aliquot of the in vitro translation reaction; 14% of binding reaction input. MBP-Axin/MBP levels were detected by SDS-PAGE and anti-MBP Western blotting of 1% of the input.

dependent on GBP binding to Xgsk-3, the coimmunoprecipitation experiment was repeated using a high dose of HA-tagged GBP (GBPwt) or an equivalent amount of the HA-tagged GBP mutant that does not bind Xgsk-3 (Yost et al., 1998) (GBPmut). The level of Axin coimmunoprecipitated by Xgsk-3 was reduced by expression of GBPwt but not by GBPmut (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 (298-506), which binds GSK-3 as well as full-length Axin (Ikeda 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) (Ikeda et al., 1998). 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 effec-

tively binds [^{35}S]GSK-3 (lane 6), it does not interact with [^{35}S]GBP (Fig. 2 b, lane 4). Moreover, MBP-Axin does not associate with [^{35}S]GBP when [^{35}S]GSK-3 is present (Fig. 2 b, 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 Axin cannot bind GSK-3 simultaneously suggests that GBP might inhibit GSK-3 by removing it from the Axin complex or by preventing GSK-3 from associating with Axin. 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* (Yost et al., 1998) RNA and, 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 uninjected embryos were immunoprecipitated with both antibodies to measure background. The immunoprecipitates were incubated with γ-[^{32}P]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 (dnXgsk-3) in *Xenopus* (Dominguez et al., 1995; He et al., 1995; Pierce and Kimelman, 1995). The demonstration that Axin binds GSK-3 and promotes the phosphorylation of β-catenin suggests that the dnXgsk-3 might function by binding Axin and keeping it from binding endogenous Xgsk-3. However, studies in a mammalian system have shown that kinase dead GSK-3 does not bind Axin (Ikeda et al., 1998), indicating that the kinase dead dnXgsk-3 might stabilize β-catenin by an alternative mechanism. To investigate this issue, we compared Xgsk-3 and dnXgsk-3 binding to Axin in coimmunoprecipitation experiments. *Axin-myc* was coinjected with either *Xgsk-3-FLAG* or *dnXgsk-3-FLAG* in *Xenopus* embryos, and expressed proteins were immunoprecipitated with anti-FLAG antibodies. Immunocomplexes were analyzed by Western blotting with anti-myc and anti-FLAG antibodies. As expected, Axin is immunoprecipitated by Xgsk-3 (Fig. 4, lane 7). In addition, Axin is immunoprecipitated equally well by dnXgsk-3 (Fig. 4, lane 8). Thus, unlike in the mammalian system, the kinase dead mutant of Xgsk-3 binds Axin, suggesting that it stabilizes β-catenin either by displacing endogenous Xgsk-3 from the Axin complex, or by preventing association of Axin and endogenous Xgsk-3.

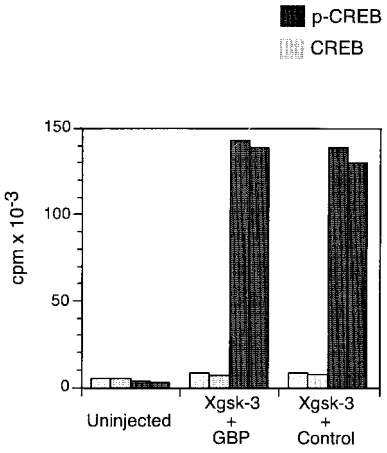

Figure 3. GBP does not inhibit Xgsk-3 phosphorylation of a peptide substrate. Embryos were injected with RNA encoding *Xgsk-3-myc* together with control RNA or *GBP-FLAG* RNA. After 3 h, proteins were extracted and immunoprecipitated with anti-FLAG (Xgsk-3 + GBP), anti-myc (Xgsk-3 + control), or both (uninjected) antibodies. The kinase activity of immune complexes was measured by phosphorus-32 incorporation into the GSK-3–specific substrate prephosphorylated CREB peptide (p-CREB; dark bars). The nonphosphorylated CREB peptide (CREB; light bars) is not a GSK-3 substrate and was used as a control. The activity of duplicate immune complexes is shown.

Truncated APC Stabilizes β-Catenin

Various lines of evidence have implicated APC in the downregulation of β-catenin. In the colon cancer cell line SW480, for example, a mutation that truncates APC in the

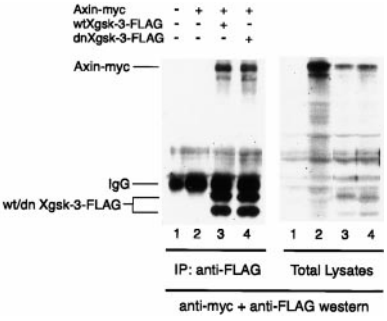

Figure 4. Dominant-negative Xgsk-3 binds Axin. Embryos were injected at the two- to eight-cell stage with 1 ng *Axin-myc*, 0.5 ng *Xgsk-3-FLAG*, and 0.5 ng *dnXgsk-3-FLAG* in the animal pole. Embryo extracts were precipitated with anti-FLAG antibody and detected by Western blotting (left panel). An aliquot of each sample taken before immunoprecipitation is shown in the right panel (Total Lysates). Lane numbers in the right panel refer to the same injections as shown above corresponding lane numbers in the left panel.

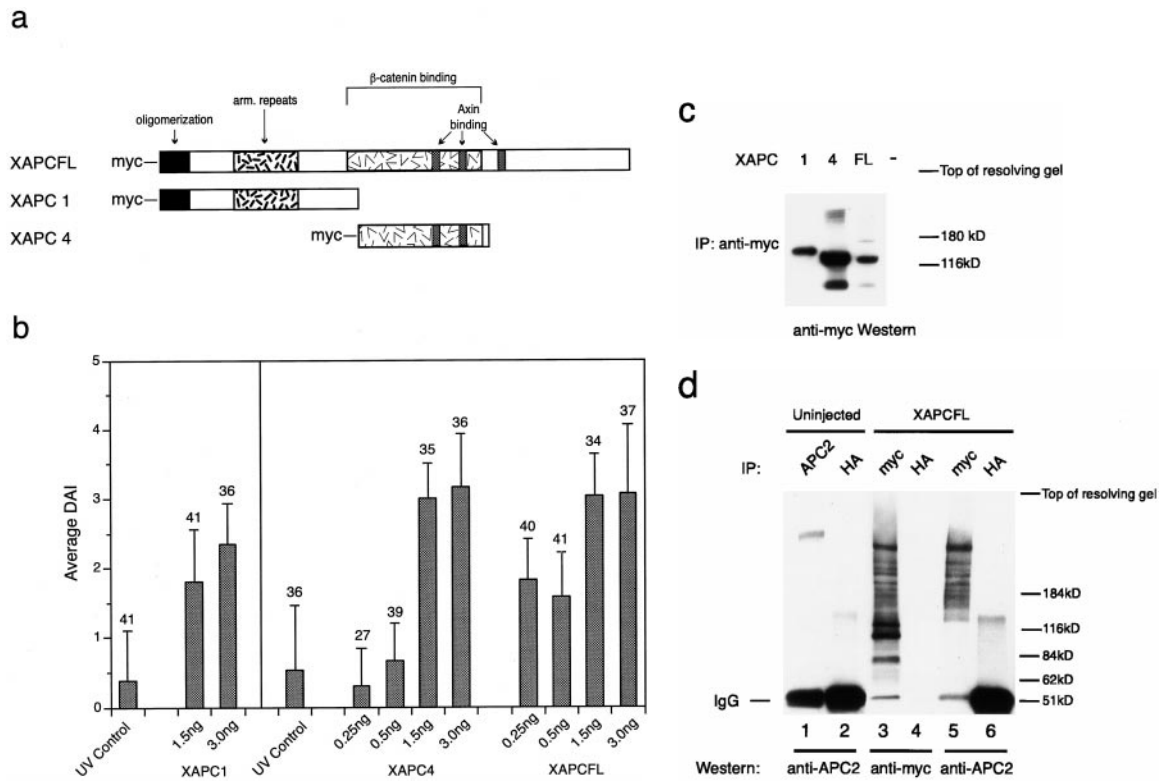

Figure 5. Regulation of β-catenin levels by *Xenopus* APC. (a) XAPC constructs used in axis rescue and β-catenin stabilization experiments. (b) XAPC induces an axis in *Xenopus*. In two separate experiments, RNA encoding different forms of XAPC was injected at the doses indicated into the marginal zone of UV-irradiated embryos and the degree of axis rescue was determined at tadpole stages. An unaffected embryo has a DAI of 5 and a completely ventralized embryo has a DAI of 0. Uninjected ventralized UV control embryos are indicated for each experiment. Bars indicate SD and numbers above bars indicate the number of embryos scored for each injection. (c) Truncated forms of XAPCFL result from its overexpression in *Xenopus* embryos. RNA encoding myc-tagged versions of XAPC were injected into cleavage stage embryos. After a 4-h incubation, embryo extracts were collected and immunoprecipitated with anti-myc antibody. After SDS-PAGE, protein products were detected by Western blot with anti-myc antibodies. (d) The observed truncated products are present in the intact embryo. Uninjected embryos and sibling embryos injected with 5.6 ng myc-tagged *XAPCFL* were immunoprecipitated with anti-APC2, anti-myc, or anti-HA (negative control) antibody as indicated. After SDS-PAGE, APC was detected using anti-APC2 or anti-myc antibody, as indicated. Endogenous APC is not detected in the sample injected with XAPCFL (lanes 3–6) because this sample was not immunoprecipitated with the APC2 antibody. The lower molecular mass forms of XAPCFL are not observed with the anti-APC2 antibody which binds to the central region of APC.

20–amino acid repeat region leads to the accumulation of high levels 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 NH_2 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 NH_2 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 Vleminckx 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 NH_2-terminal third or less of APC. Because endogenous APC appeared undegraded, 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 Vleminckx 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 Vleminckx 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 (*GFP*) 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 Vleminckx 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 NH_2-terminal

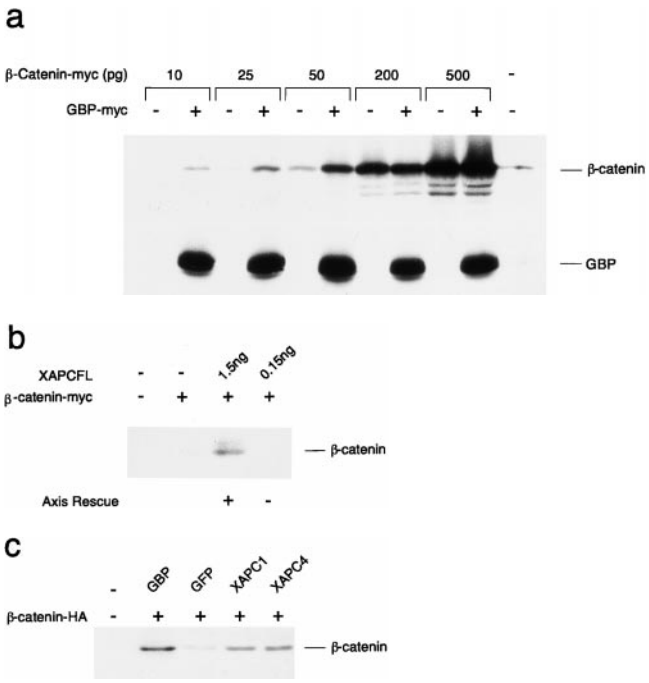

Figure 6. Truncated APC products cause axis duplication through β-catenin stabilization. Detection of β-catenin-myc stabilization depends upon the dose of RNA injected. Embryos were injected at the two- to eight-cell stage in the animal pole with the indicated dose of β-*catenin-myc*, with or without 1 ng *GBP-myc* as indicated, and with a control RNA (*GFP*) to yield a total of 2 ng of injected RNA per treatment. Embryos were lysed at stage 8 and approximately one embryo equivalent from each treatment was analyzed by Western blotting with the anti-myc antibody. (b) Ectopic XAPC stabilizes β-catenin. Embryos were injected with 50 pg of RNA encoding myc-tagged β-catenin alone or together with *XAPCFL* RNA (0.15 or 1.5 ng). Control embryos were uninjected. After 4 h, protein samples were collected for Western analysis and β-catenin-myc was detected with anti-myc antibody. In parallel experiments, the ability of these RNAs to induce an axis in UV irradiated embryos was measured. (c) XAPC1 and XAPC4 stabilize β-catenin. Embryos were injected with 50 pg of RNA encoding HA-tagged β-catenin together with 2 ng *GBP* (positive control), *GFP* (negative control), *XAPC1*, or *XAPC4* RNA. After 4 h, protein samples were collected for Western analysis and β-catenin-HA was detected with anti-HA antibody.

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 Dishevelled, 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* (Dominguez et al., 1995; He 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 (Hart et al., 1998; Ikeda et al., 1998; Itoh et al., 1998; Nakamura et al., 1998; Sakanaka et al., 1998; Yamamoto et al., 1998), and promotes the phosphorylation of β-catenin by GSK-3 (Hart et al., 1998; Ikeda et al., 1998; Yamamoto 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 coimmunoprecipitated 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 GSK-3 activity can be controlled by its preferential binding to either GBP or Axin.

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 $p21^{Cip1,WAF-1}$, $p27^{Kip1}$, and $p57^{Kip2}$. When $p27^{Kip1}$ binds to Cdk2, it causes large structural changes in the NH_2 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 $p27^{Kip1}$ 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 Xgsk-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 (Dominguez et al., 1995; He 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-3Y>F also coimmunoprecipitates less Axin than wild-type GSK-3 in mammalian cells (Yuan et al., 1999). Furthermore, Akt, which inhibits GSK-3 by phosphorylating it, reduces the amount of Axin coimmunoprecipitated 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 phosphorylates both APC and β-catenin as a prerequisite for β-catenin degradation (Rubinfeld et al., 1996). Data obtained from a study overexpressing APC in *Xenopus*, 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 *Xenopus* 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 nucleus 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 APR-1 positively regulates WRM-1 (Rocheleau et al., 1997).

Our results support a model in which APC acts to downregulate β-catenin in *Xenopus*. We found that injection of RNA encoding full-length *APC* (*XAPCFL*) in *Xenopus* 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; Yost et al., 1996), dnXgsk-3 (Yost et al., 1996), or GBP (Yost et al., 1998) are overexpressed in *Xenopus* embryos. No alternate or parallel pathway needs be invoked to explain the axis-inducing effect of ectopically expressed APC in *Xenopus*.

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 *Xenopus* 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 Axin binding domains identified in APC (Behrens et al., 1998; Hart et al., 1998), and is likely to displace endogenous APC from the Axin–Xgsk-3 complex, preventing it from participating in the degradation of β-catenin. Supporting this interpretation, it has been shown that an APC 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 APC, present in *Xenopus* but not in SW480 cells, or that more protein may be produced by overexpression in *Xenopus* than transfection of mammalian cells. Unlike Vleminckx et al. (1997), we also observed axis rescue by XAPC1, which is the most NH_2-terminal fragment and lacks the identified Axin binding domains (Fig. 5 a). This fragment contains an NH_2-terminal oligomerization domain (Su et al., 1993), which could bind the endogenous APC and block its function, and also a domain of Arm repeats, which might be expected to mediate an interaction between APC and an unidentified partner.

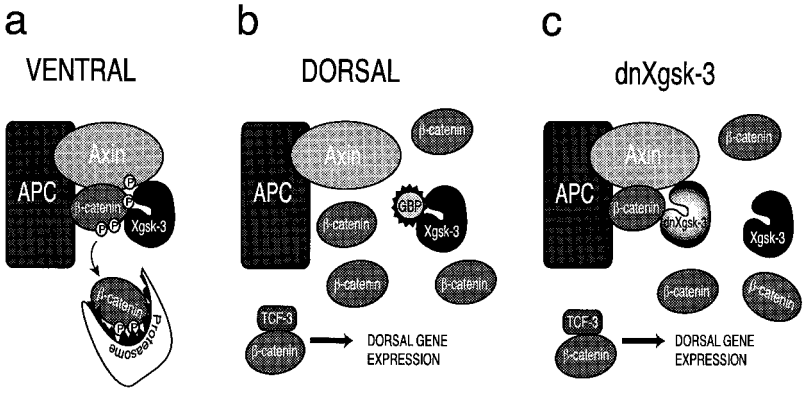

Figure 7. Model for GSK-3 regulation in the early *Xenopus* embryo. (a) On the ventral side of the embryo, GSK-3 is part of a functional degradation complex that includes APC and Axin. When in this complex, GSK-3 phosphorylates β-catenin, targeting it for degradation via the proteosome pathway. Under these conditions, dorsal genes are repressed. (b) On the dorsal side of the embryo, GSK-3 is excluded from the Axin–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 Axin, thus, keeping endogenous Xgsk-3 from the degradation complex. As in the situation with GBP, β-catenin accumulates 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 Yost et al., 1997). The experiments presented here advance our understanding of GSK-3 regulation and allow us to propose the following model for Xgsk-3 regulation in the fertilized *Xenopus* egg. On the ventral side, β-catenin levels are kept low by Xgsk-3–dependent phosphorylation in a complex including Axin and APC (Fig. 7 a). Phosphorylation of Axin and APC by Xgsk-3 may be required to assemble this complex. On the dorsal side, GBP inhibits phosphorylation of β-catenin either by displacing Xgsk-3 from the Axin/APC/Xgsk-3 complex or by prebinding GSK-3 and preventing its association with the Axin–APC complex. GBP binding to Xgsk-3 may block access of protein substrates to the active site (Fig. 7 b). GBP might also prevent Xgsk-3 from phosphorylating Axin and APC, and thereby further inhibit complex formation. The dominant-negative Xgsk-3 acts in a manner analogous to GBP since it either displaces wild-type Xgsk-3 from the complex, or binds endogenous Xgsk-3 and prevents its binding to Axin, 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 very interesting to elucidate the relative dorso-ventral abundance of each of these proteins in the *Xenopus* oocyte and fertilized embryo. However, it may not be just the relative abundance of each of these players that specifies the endogenous axis. Alternatively, posttranslational modifications and/or the presence of yet unidentified factors could affect dorso-ventral differences in known players. GBP is a candidate for the dorsal determinant, and we are developing the reagents to examine the possible dorsal localization or dorsal modification of GBP. Alternatively, GBP may be ubiquitous, but be activated or recruited to function by another factor that becomes dorsally localized in response to sperm entry. The determinant might, for example, bind to the Axin/APC/Xgsk-3 complex and alter its conformation in such a way as to allow GBP to bind Xgsk-3 and remove it from the complex. In this regard, the observation that Dishevelled interacts with GBP (Li et al., 1999) and Axin (Kishida et al., 1999; Smalley et al., 1999) and is dorsally enriched in the early *Xenopus* embryo (Miller et al., 1999) is very suggestive of such a mechanism. Alternatively, GBP might function together with Dishevelled to prevent the initial formation of complexes between Axin and GSK-3 in the dorsal cortical cytoplasm, rather than functioning to disrupt complexes once they have formed.

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Note Added in Proof. While this work was under review, a study was published showing that a peptide derived from the GSK-3 binding region of FRAT1 prevents the association of Axin and GSK-3 in agreement with the results presented here (Thomas, G.M., S. Frame, M. Goedert, L. Nathke, P. Polakis, and P. Cohen. 1999. *FEBS (Fed. Eur. Biochem. Soc.) Lett.* 458:247–251.

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