Regulation of Spemann organizer formation by the intracellular kinase Xgsk-3

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SUMMARY

Dorsal axis formation in the Xenopus embryo can be induced by the ectopic expression of several Wnt family members. In Drosophila, the protein encoded by the Wnt family gene, wingless, signals through a pathway that antagonizes the effects of the serine/threonine kinase zeste-white 3/shaggy. We describe the isolation and characterization of a Xenopus homolog of zeste-white 3/shaggy, Xgsk-3. A kinase-dead mutant of Xgsk-3, Xgsk-3K→R, has a dominant negative effect and mimics the ability of Wnt to induce a secondary axis by induction of an ectopic Spemann organizer. Xgsk-3K→R, like Wnt, induces dorsal axis formation when expressed in the deep vegetal cells, which do not contribute to the axis. These results indicate that the dorsal fate is actively repressed by Xgsk-3, which must be inactivated for dorsal axis formation to occur. Furthermore, our work suggests that the effects of Xgsk-3K→R are mediated by an additional intercellular signal.

Key words: Xenopus, Spemann organizer, kinase XGSK-3, dorsal axis formation, Wnt

INTRODUCTION

Pattern formation in the early Xenopus embryo involves a series of intercellular signaling events which occur during the early cleavage stages. With the identification of many of the key signaling factors, it has become increasingly clear that they are likely to act in combination to regulate early inductive events (Kimelman et al., 1992; Sive, 1993; Cornell and Kimelman, 1994b). A member of the fibroblast growth factor (FGF) family and an activin-like molecule, perhaps Vg1, appear to act together to induce mesoderm to form at the equator of the embryo (R. Cornell, T. Musci, and D. Kimelman, unpublished data). During this same time period, a dorsaling signal, present in the dorsal vegetal and perhaps dorsal marginal zone blastomeres, acts to induce the dorsal organizing center, known as the Spemann organizer, in the dorsal mesoderm (Gimlich and Gerhart, 1984). The Spemann organizer then generates signals that dorsalisize the adjacent mesoderm, creating the dorsoventral axis (Spemann and Mangold, 1924). Several factors have been proposed to be the dorsaling signal that induces the Spemann organizer, including members of the Wnt family, noggin, and high levels of Vg1, all of which have the ability to induce ectopic axis formation when expressed in the ventral region of the early embryo (Smith and Harland, 1991, 1992; Sokol et al., 1991; Thomsen and Melton, 1993).

Although many of the signaling factors involved in dorsoventral patterning have been identified, little is known about the intracellular signaling mechanisms by which they act. FGFs act via a MAP kinase-dependent pathway, whereas activation-mediated mesoderm induction occurs through a different and, as of yet, unknown pathway (Graves et al., 1994; Hartley et al., 1994; LaBonne and Whitman, 1994). Receptors for Wnt and noggin have not been identified, and hence these pathways are poorly understood. Lithium chloride, which causes embryos to develop an ectopic axis (Kao et al., 1986), appears to act by disrupting the phosphoinositide (PI) cycle (Basa and Gimlich, 1989; Maslanski et al., 1992), suggesting that PI turnover may be an important aspect of the intracellular signaling pathways stimulated by dorsaling factors. In addition, there is enhanced gap junction activity on the dorsal side of the 32-cell embryo (Guthrie, 1984), an effect that can be produced on the ventral side of embryos by the expression of Xwnt-8 (Olson et al., 1991) or the addition of lithium chloride (Nagajskie et al., 1989), indicating that intercellular communication through gap junctions may be involved in early patterning of the dorsoventral axis (Warner et al., 1984; Guthrie et al., 1988).

In a few cases, large parts of intracellular signaling pathways are conserved between species, even though the signals are used to accomplish different objectives (Nishida and Gotoh, 1993). The Drosophila gene wingless (wg) encodes a member of the Wnt family, which has axis-inducing properties when expressed in the Xenopus embryo (Chakrabarti et al., 1992). Several genes have been identified that potentially act downstream of wg, including zeste-white 3/shaggy (zw3/shaggy), armadillo and dishevelled (Siegfried et al., 1992, 1994; Noordermeer et al., 1994). Zw3/shaggy encodes a cytoplasmic Ser/Thr kinase whose effects are antagonized by wg signaling (Bourouis et al., 1990; Siegfried et al., 1992). The mammalian homologs of zw3/shaggy, glycogen synthase kinase-3α and β (GSK-3α and β), have recently been shown to be regulated by targets of growth factor signaling (Sutherland et al., 1993), and zw3/shaggy/GSK-3 homologs have also been identified in yeast and plants (Bianchi et al., 1993, 1994; Pay et al., 1993;
A frame-shift mutant of Xgsk-3 (Xgsk-3FS) was made by digesting pXG30 with EcoRV, which cleaves in the middle of the Xgsk-3 gene, ligating in the presence of BglII linkers (8-mer; New England Biolabs), digesting with BglII and then ligating to recircularize the plasmid. This created an insertion resulting in a frame-shift mutation.

Construction of the Xgsk-3 kinase-dead mutant

The kinase-dead mutant of Xgsk-3 (Xgsk-3K→R) was constructed using a PCR-based overlap extension method (Ho et al., 1989). Complimentary primers were designed to cover the region to be mutated, incorporating a single nucleotide change to convert a lysine to an arginine residue. These primers, GSK-K85R-F and GSK-K85R-R, had the sequences 5′-CGCGGAAGGTCCGGG-3′ and 5′-CGCGGTACCCAGTGCT-3′, respectively. 67 DNA was used as the template in two separate PCR reactions with the primer pairs GSK-F/GSK-K85R-R and GSK-K85R-F/GSK-R. The resulting overlapping fragments were purified on agarose gels and used together as the template in a PCR reaction with GSK-F and GSK-R as primers. PCR conditions for these reactions were 2 cycles at 94°C for 1 minute, 42°C for 3 minutes, and 72°C for 1.5 minutes; 15 cycles at 94°C for 1 minute, 60°C for 1 minute and 72°C for 1.5 minutes. The full-length amplified fragment with the incorporated mutation was purified on an agarose gel, digested with BamHI and inserted into the BamHI site of a BluescriptII KS′ vector, creating pXG21 and into the BglII site of a pSP64T vector, creating pXG40. The presence of the correct mutation was confirmed by nucleotide sequence analysis.

Embryos

Fertilized embryos were obtained as previously described (Newport and Kirschner, 1982). Eggs were fertilized in 0.5× MMR (1× MMR is 0.1 M NaCl, 2.0 mM KCl, 1.0 mM MgSO4, 2.0 mM CaCl2, 0.1 mM EDTA and 5.0 mM HEPES, pH 7.8). The jelly coat was removed with 2% cysteine in water, pH 7.8, and eggs were rinsed in 0.1× MMR. Embryos were kept at 14°C to 23°C. Staging was as previously described (Newkoo and Faber, 1967). For dissection experiments, the dorsal side of embryos was marked with Nile blue at the 4-cell stage.

Animal caps

The upper portion of the animal hemisphere was dissected from stage 9 embryos with a fine wire knife. Care was taken to remove any adherent vegetal cells. Caps were cultured in 1× MMR with 1 mg/ml RMMR. Embryos were kept at 14°C to 23°C. Staging was as previously described (Newkoo and Faber, 1967). For dissection experiments, the dorsal side of embryos was marked with Nile blue at the 4-cell stage.

RNA synthesis and microinjection

Xgsk-3, Xgsk-3K→R, and Xgsk-3FS RNA was synthesized from the pSP64T vector, linearized with BamHI. AΔgsk-3 RNA was synthesized from pXG30 linearized with Smal, which produces a form of the Xgsk-3 protein that is truncated within the kinase domain. The template for β-galactosidase RNA was CS-β-galactosidase (Turner and Weintraub, 1994), linearized with NotI. RNA was synthesized using the SP6 mMESSAGE mMACHINE kit (Ambion) following the manufacturer’s instructions. Phenol:chloroform (1:1) extracted RNA was separated from unincorporated nucleotides with a Microcon 100 microcentrator (Amicon) and injected without further purification. RNA was microinjected as published (Moon and Christian, 1989), in volumes of 10 nl or less per blastomere.

RNA isolation and RNase protection

RNA was prepared by homogenization in an SDS-protease K buffer (Cornell and Kimelman, 1994a) and analyzed by the RNase protection assay (Melton et al., 1984). For Xgsk-5, 20 μg of whole embryo RNA or RNA isolated from 10 dissected embryos was used. For muscle actin, 2.5 animal cap equivalents of RNA were used. The muscle actin and EF-1α genes were used as previously described (Cornell and Kimelman, 1994a). To make the Xgsk-3 probe, a BamHI-
Fig. 1. Amino acid sequence of Xgsk-3. The predicted amino acid sequences of Xgsk-3, GSK-3β, and zw3/shaggy were compared, with non-identical residues in GSK-3β and zw3/shaggy indicated. The putative kinase region encompasses residues 54 to 325. The lysine residue which may indicate an evolutionary relationship among these three genes.

In order to determine whether the Xgsk-3 transcript is present during the blastula stages, when mesoderm is induced and patterned (Jones and Woodland, 1987), RNA was isolated from early Xenopus embryos and analyzed by the RNase protection assay. Using a probe derived from the 5' end of the Xgsk-3 cDNA, Xgsk-3 was found to be expressed at a relatively constant level in the unfertilized egg and throughout the early embryonic stages (Fig. 2A), demonstrating that Xgsk-3 is present in the embryo as a maternal transcript. To determine whether the maternal Xgsk-3 transcripts are localized in the Xenopus embryo, RNA was isolated from dissected embryos and analyzed by the RNase protection assay. When 32-cell-stage embryos were dissected into dorsal and ventral halves, Xgsk-3 was found to be expressed at the same level in both halves (Fig. 2B, lanes 1 and 2). Stage 9 embryos were dissected into dorsal and ventral halves or the animal hemisphere was dissected away from the rest of the embryo. At stage 9, Xgsk-3 was also found to be expressed at equal levels in all parts of the embryo (Fig. 2B, lanes 3-6).

A kinase-dead mutant of Xgsk-3 causes axis duplication

In order to explore the function of Xgsk-3, we generated a kinase-dead mutant of Xgsk-3. All kinases have a conserved lysine residue in the ATP-binding region, which is necessary for kinase activity (Hanks et al., 1988). Conversion of this lysine residue to another amino acid abolishes kinase activity and, in some cases, results in the creation of a dominant negative mutant (MacNicol et al., 1993). With the hope of creating a dominant negative mutant of Xgsk-3, we used a PCR-based strategy to change a single nucleotide in the Xgsk-3 cDNA, converting the conserved lysine to an arginine residue (Fig. 1). This mutant is referred to as Xgsk-3K→R.

The function of Xgsk-3K→R was investigated by ectopic overexpression in embryos. Injection of 0.5 ng per blastomere of RNA encoding Xgsk-3K→R into the lateral sides of 2-cell embryos resulted in tadpoles with two heads, indicating duplication of the anterior dorsal axis (Fig. 3). Although some variability was observed when embryos from different mothers were used, the duplicated axis phenotype was typically observed in 10% of the laterally injected embryos. 20% of the embryos were normal while the remaining 70% exhibited various axial and dorsoanterior defects. When the site of
injection was confined to the future ventral side, the percentage of embryos with axis duplication went up to 86%, while little effect was seen when Xgsk-3K→R RNA was injected on the future dorsal side (>90% of the embryos were normal). The duplicated axis phenotype was somewhat variable, most commonly including two well-formed heads and a shortened body (Fig. 3C). Less commonly, the embryos had two perfectly formed heads and a normal trunk and tail (Fig. 3B) or one head with widened or fused features (data not shown). At higher doses of injected RNA, the resulting embryos were severely dorsoanteriorized (data not shown). Since injection of RNA encoding a kinase-dead version of Xgsk-3 resulted in a specific phenotype, we hypothesized that Xgsk-3K→R acts as a dominant negative mutant, and thus was interfering with a normal developmental pathway involved in dorsal axis formation.

Xgsk-3K→R, like Xwnt-8, rescues dorsal axis formation in UV-irradiated embryos

Dorsal axis duplication can also be caused by ectopic overexpression of members of the Wnt family, including Xwnt-8 (Christian et al., 1991; Sokol et al., 1991). Since the Xgsk-3 homolog, zw3/shaggy, is involved in signaling by the Wnt family member, wnt, in Drosophila (Siegfried et al., 1992, 1994; Diaz-Benjumea and Cohen, 1994), we hypothesized that Xgsk-3K→R RNA might mimic another known effect of Xwnt-8. If injection of Xgsk-3K→R RNA had an average DAI of 4.0 (Fig. 4D). Therefore, Xgsk-3K→R, like Xwnt-8, can induce the formation of a normal dorsal axis in a ventralized embryo.

Wild-type Xgsk-3 opposes the Xgsk-3K→R phenotype

If Xgsk-3K→R is having a dominant negative effect and interfering with the action of endogenous Xgsk-3, we expected that the addition of excess Xgsk-3 would be able to overcome the effect of Xgsk-3K→R. This was tested by asking whether injection of Xgsk-3K→R RNA could prevent dorsal axis rescue by Xgsk-3K→R in UV-irradiated embryos. Embryos were UV-irradiated as described above and injected with 0.5 ng of Xgsk-3K→R RNA, or with 0.5 ng of Xgsk-3K→R RNA in combination with 1 ng of Xgsk-3 RNA. After 3 days, the DAI of the embryos was scored. Injection of RNA encoding a frameshift mutant of Xgsk-3, that produces a phenotype indistinguishable from that of uninjected embryos or embryos injected with ΔXgsk-3 RNA, did not rescue the dorsal axis and...
the resulting embryos had an average DAI of 0.7 (n=32). In contrast, UV-irradiated embryos injected with $Xgsk-3K\rightarrow R$ RNA had an average DAI of 3.3 (n=30), indicating restoration of dorsal axis formation. Co-injection of a two-fold excess of $Xgsk-3$ RNA completely blocked the rescuing activity of $Xgsk-3K\rightarrow R$, producing embryos with an average DAI of 1.0 (n=30). These results indicate that $Xgsk-3K\rightarrow R$ functions by interfering with endogenous $Xgsk-3$.

**Effect of $Xgsk-3$ and $Xgsk-3K\rightarrow R$ on $goosecoid$ and $Xnot$, markers of prospective dorsal mesoderm**

We reasoned that the effects on dorsal axis development caused by overexpression of $Xgsk-3K\rightarrow R$ should be reflected in the early expression of dorsal specific genes if $Xgsk-3$ is involved in the early patterning events. Embryos were injected ventrally with 2 ng of $Xgsk-3K\rightarrow R$ RNA and cultured until the early gastrula (stage 10) or late gastrula (stage 12) stages. In addition, to test the effects of excess $Xgsk-3$ on the dorsal side, embryos were injected dorsally with 4 ng of $Xgsk-3$ RNA and cultured until stage 10 or stage 12. The embryos were fixed and the expression of the head-specific and notochord-specific homeobox genes $goosecoid$ ($gsc$) and $Xnot$ was determined by in situ hybridization. $Gsc$ is expressed at stage 10 in a patch above the dorsal lip, marking the future head mesoderm (Cho et al., 1991). Uninjected embryos (Fig. 5A) and embryos injected either dorsally or ventrally with $\Delta Xgsk-3$ RNA (data not shown) showed the normal pattern of expression. Embryos injected ventrally with $Xgsk-3K\rightarrow R$ RNA typically had two dorsal lips at this stage with $gsc$ expressed in a patch above each of them (Fig. 5B). In contrast, over 50% of the embryos injected dorsally with $Xgsk-3$ RNA have not begun to gastrulate at this stage and did not have visible $gsc$ staining (Fig. 5C). In the remaining embryos, $gsc$ staining was present but was fainter than in control embryos. At stage 12, $Xnot$ is expressed in the presumptive notochord (von Dassow et al., 1993), as seen in uninjected embryos (Fig. 5D) and in embryos injected either dorsally or ventrally with $\Delta Xgsk-3$ RNA (data not shown). In addition to causing duplication of $gsc$ expression, ventral injection of $Xgsk-3K\rightarrow R$ RNA resulted in duplication of $Xnot$ expression (Fig. 5E). Embryos injected dorsally with $Xgsk-3$ RNA generally showed $Xnot$ staining, but the region of staining was not as tightly defined and was generally fainter than in uninjected embryos (Fig. 5F). These results demonstrate that the effects of $Xgsk-3K\rightarrow R$ are due to early perturbations of mesodermal patterning.

Although $Xwnt-8$ can induce the formation of a secondary axis in the *Xenopus* embryo and can induce the expression of mesodermal genes such as $Xnot$ (von Dassow et al., 1993), it cannot directly induce mesoderm (Christian et al., 1992). However, $Xwnt-8$ has been shown to synergize with bFGF in the induction of dorsal mesoderm (Christian et al., 1992), suggesting that it acts primarily to regulate the type of mesoderm that forms. To determine whether $Xgsk-3K\rightarrow R$ shares these properties with $Xwnt-8$, the mesoderm-inducing properties of $Xgsk-3K\rightarrow R$ were examined in an animal cap assay. The upper region of the animal hemisphere (the animal cap) was
explanted at stage 9 from uninjected embryos or embryos injected in the animal pole with 1 ng of Xgsk-3K→R RNA at the 2-cell stage. The animal caps were then cultured with or without bFGF for approximately 20 hours. RNA extracted from the caps was analyzed by the RNase protection assay using a probe for the mesoderm marker, muscle actin. Animal caps from uninjected or Xgsk-3K→R RNA-injected embryos express no muscle actin when cultured without bFGF (Fig. 2C, lanes 1 and 3), but muscle actin is strongly induced in the presence of bFGF (Fig. 2C, lanes 2 and 4). The level of muscle actin transcript induced in Xgsk-3K→R-expressing animal caps is approximately 3-fold greater than in animal caps from uninjected embryos. These results demonstrate that Xgsk-3K→R is not able to directly induce mesoderm, and, like Xwnt-8, it can synergize with bFGF in the induction of mesoderm.

Xgsk-3K→R rescues dorsal axis formation from the deep vegetal cells

The deep vegetal cells of the *Xenopus* embryo are able to induce the formation of the Spemann organizer in the marginal zone, even though they do not become incorporated into the mesoderm (Gimlich and Gerhart, 1984). Similarly, Xwnt-8 can induce the formation of a complete dorsal axis when expressed in deep vegetal cells of embryos ventralized by exposure to UV light, even though none of the cells expressing Xwnt-8 become part of the induced axis (Smith and Harland, 1991). It is believed that these results are due to the ability of Xwnt-8 protein to be secreted from the deep vegetal cells and therefore to alter the fate of the overlying marginal zone cells. Since we expected that Xgsk-3 was an intracellular target of a Wnt-like signaling pathway, based on the studies of *wingless* and *shaggy* in *Drosophila* (Siegfried et al., 1992; Diaz-Benjumea and Cohen, 1994), we predicted that Xgsk-3K→R would not be able to induce axis formation when expressed in the deep vegetal cells.

UV-irradiated 32-cell embryos were selectively injected in the marginal zone (tier C) or deep vegetal cells (tier D) with Xgsk-3K→R RNA in combination with RNA encoding β-galactosidase, to identify the cells expressing the injected RNA (Smith and Harland, 1991). As shown in Fig. 6, Xgsk-3K→R was able to rescue dorsal axis formation when expressed in either tier C or tier D cells (Fig. 6B,C). These results were quantitated by scoring the DAI of the embryos.

**Fig. 4.** Xgsk-3K→R rescues dorsal axis formation in UV-irradiated embryos. Fertilized eggs were UV-irradiated for 60 seconds within 40 minutes after fertilization. At the 4-cell stage, one cell was injected with the indicated RNA and the embryos were allowed to develop for three days. (A) Uninjected embryo; (B) Embryo injected with 2 ng ΔXgsk-3 RNA; (C) Embryo injected with 1 ng Xgsk-3K→R RNA; (D) The dorsoanterior index (DAI) of the uninjected (n=54) (upper panel) and Xgsk-3K→R RNA injected (n=22) (lower panel) embryos was scored. The percentage of embryos with each score is shown.
Regulation of axial pattern by Xgsk-3

β-galactosidase staining confirmed that tier C cells contribute to the induced axis while tier D cells contribute only to endoderm (Fig. 6B,C). Xgsk-3K→R RNA was somewhat less effective at rescuing dorsal axis formation when injected at the 32-cell stage than when injected at the 4-cell stage (Fig. 4). These results indicate that elimination of Xgsk-3 signaling either from the cells that contribute to Spemann’s organizer, or from the deep vegetal cells that do not contribute to the organizer, is sufficient to induce the formation of a dorsal axis.

DISCUSSION

We have described the isolation of a Xenopus homolog of the mammalian GSK-3 and Drosophila zw3/shaggy genes, Xgsk-3, which is involved in dorsoventral patterning in the early embryo. The amino acid sequence of Xgsk-3, like zw3/shaggy, has a greater degree of identity to GSK-3β (92%) than to GSK-3α (75%). The high degree of sequence conservation between Xgsk-3, zw3/shaggy and GSK-3β suggests a possible conserved role for these proteins among flies, frogs and mammals. Xgsk-3 is expressed as a maternal transcript, which is present throughout the early embryonic stages, consistent with a role for Xgsk-3 in early patterning events, which are thought to take place during the blastula stages. Analysis of dissected embryos shows that the Xgsk-3 transcript is present at equal levels throughout the embryo. In situ hybridization experiments also indicate that Xgsk-3 transcripts are not localized during the blastula stages, but instead are uniformly distributed throughout the embryo (unpublished results), although we cannot entirely exclude the possibility that Xgsk-3 transcripts are absent from a small area of the embryo.

Induction of the Spemann organizer by a kinase-dead mutant of Xgsk-3

Genetic studies in Drosophila have defined a role for zw3/shaggy in the wg signaling pathway. During embryonic segmentation, wg signaling is required for the maintenance of en entrainment in a group of adjacent cells (Bejsovec and Martinez-Arias, 1991). In loss-of-function zw3/shaggy mutant embryos, the region of en expression is expanded, and its maintenance is independent of wg signaling (Siegfried et al., 1992). These results have led to a model in which the regulation of en expression by Wg is mediated by the inactivation of zw3/shaggy (Siegfried et al., 1992). Similarly, the inactivation of zw3/shaggy by wg signaling has been proposed to be necessary for the specification of the ventral cells in the Drosophila leg (Diaz-Benjumeda and Cohen, 1994). If this mechanism of action has been conserved by the Wnt signaling pathway in Xenopus, we would predict that Xwnt-8 signaling would result in the inactivation of the zw3/shaggy homolog, Xgsk-3. To test this prediction, we constructed a kinase-dead version of Xgsk-3, Xgsk-3K→R, with the hope that it would act as a dominant negative mutant.
When RNA encoding Xwnt-8 is injected into the ventral side of a Xenopus embryo, a second dorsal axis is formed due to the induction of an ectopic Spemann organizer (Smith and Harland, 1991; Sokol et al., 1991). Ventral injection of RNA encoding Xgsk-3K→R also resulted in dorsal axis duplication, suggesting that Xgsk-3 acts on the same pathway as Xwnt-8. This conclusion is supported by the ability of Xgsk-3K→R to rescue dorsal axis formation in embryos ventralized by UV-irradiation. In addition, like Xwnt-8, Xgsk-3K→R has no effect on normal dorsal axis formation when it is ectopically expressed on the dorsal side of the embryo. Since expression of a kinase-dead form of Xgsk-3 caused a specific phenotype, and since addition of excess wild-type Xgsk-3 eliminated the effects of Xgsk-3K→R, we conclude that Xgsk-3 acts as a dominant negative mutant by interfering with the function of endogenous Xgsk-3. Xgsk-3K→R may act as a dominant inhibitory mutant by competing with endogenous Xgsk-3 for substrates or upstream regulatory molecules. Since there is no evidence that zw3/shaggy/GSK-3 acts as a dimer, it is not likely to function by directly interfering with the endogenous Xgsk-3.

Xgsk-3 functions as an endogenous repressor of dorsal fate

Our results suggest that dorsal fate is normally repressed throughout the embryo by Xgsk-3, and that dorsal determination requires the inhibition or opposition of Xgsk-3 activity on the dorsal side of the embryo. This hypothesis is consistent with results obtained by overexpressing Xgsk-3. When Xgsk-3 is ectopically expressed on the dorsal side of the embryo, dorsal development is compromised (unpublished results). At lower doses of Xgsk-3 RNA, the resulting tadpoles have a somewhat ventralized phenotype lacking eyes, with small heads and a shortened dorsal axis. Higher doses of Xgsk-3 RNA result in complete loss of heads. However, even at high doses of Xgsk-3 RNA, the embryos are not completely ventralized, retaining some axial development, while comparatively low doses of Xgsk-3K→R RNA can completely rescue dorsal axis formation in a ventralized embryo. These results suggest that the endogenous dorsalizing signal may have the capacity to regulate much higher levels of Xgsk-3 than are normally present in the embryo.

The ability of Xgsk-3K→R to mimic Xwnt-8 suggests that the function of Xgsk-3 is necessary for one of the earliest steps of dorsoventral axis determination, the induction of the Spemann organizer. This is confirmed by the expression patterns of gsc and Xnot, two genes that are expressed in the organizer region and which mark prospective dorsal tissue. When Xgsk-3K→R is expressed on the ventral side of the embryo, the expression patterns of gsc and Xnot are duplicated, indicating duplication of the organizer itself. In contrast,
expression of Xgsk-3 on the dorsal side of the embryo alters the expression patterns of gsc and Xnot, indicating that organizer formation has been disrupted. These observations are supported by changes in the pattern of gastrulation in response to ectopic expression of Xgsk-3K→R and Xgsk-3. Expression of Xgsk-3K→R on the ventral side of the embryo causes gastrulation to initiate simultaneously on the dorsal and ventral sides of the embryo, indicating that the ventral side of the embryo has acquired the properties of the Spemann organizer. However, when Xgsk-3 is expressed on the dorsal side, gastrulation initiates later than in control ΔXgsk-3 RNA-injected embryos, suggesting that cells that would normally form the dorsal lip have acquired a more lateral quality and therefore do not begin the movements of gastrulation at the normal time.

**Xgsk-3, like zw3/shaggy, acts non-cell autonomously**

The prevailing view of Wnts when we began this project was that they acted directly as secreted morphogens to change the fate of the receiving cell and that their range of action is determined by the distance they diffuse from the signaling cell (Struhl and Basler, 1993; Thuringer and Bienz, 1993). Since zw3/shaggy is epistatic to wg (Siegfried et al., 1992), we predicted that Xgsk-3K→R would mimic the axis inducing ability of Xwnt-8 when expressed in cells that become part of the induced axis. However, since the Xgsk-3 protein is not secreted, we expected that Xgsk-3K→R would not mimic the ability of Xwnt-8 to induce axis formation when expressed in the deep vegetal cells, which do not contribute to the axis. We were therefore surprised to find that Xgsk-3K→R is as effective at rescuing axis formation in UV-irradiated embryos when expressed in the deep vegetal cells as in the marginal zone cells. There are two possible explanations for this result. If Xgsk-3 functions downstream of Xwnt-8, as predicted from the results with Drosophila, our results suggest that Xwnt-8 triggers the release of an intercellular morphogen. An alternative possibility is that Wnt signaling in Xenopus is significantly different from wg signaling in Drosophila and that Xgsk-3 is necessary for the release of an Xwnt-8-like signal, placing it upstream of Wnt in the signaling cascade. Although the second scenario is formally possible, we favor the first possibility in light of recent work in Drosophila.

Diaz-Benjumea and Cohen (1994) have found that, in the Drosophila leg imaginal disc, clones of cells that have lost the zw3/shaggy function have all the properties of clones of wg-expressing cells, including the ability to determine the fate of cells around them. This indicates that wg-expressing cells normally inhibit zw3/shaggy function in neighboring cells and that these cells in turn affect the fate of cells outside the clone. They suggest that this is due either to the release of a second secreted factor or to effects mediated by cell-cell contact. This view is supported by the results of experiments which show that Wg protein probably does not diffuse more than one cell diameter from its source (Vincent and Lawrence, 1994). Since wg signaling can be effective over distances of several cell diameters (Struhl and Basler, 1993; Thuringer and Bienz, 1993), its action may be mediated in some cases by a long-range gradient of a morphogen that is activated by Wg (Vincent and Lawrence, 1994).

Our results suggest that the emerging picture of the mechanism of wg signaling may reflect the mechanism employed by the vertebrate Wnts as well. The ability of Xgsk-3K→R to rescue dorsal axis formation from the deep vegetal cells suggests that Xwnt-8 signaling is mediated by a subsequent intercellular interaction. Although another secreted factor may be involved, an alternate possibility is that the repression of Xgsk-3 on the dorsal side of the embryo activates gap junctional communication in that region. Gap junctional activity is normally higher on the dorsal side of the embryo than on the ventral side (Guthrie, 1984). Expression of Xwnt-8 on the ventral side of the embryo enhances gap junctional communication there during the early cleavage stages (Olson et al., 1991), raising the possibility that the hypothetical long-range morphogen could pass through gap junctions.

**The function of Xgsk-3 in mesoderm induction**

Our results are consistent with the view that Xgsk-3 acts to repress an activity necessary for dorsal axis formation. Wnt (and perhaps noggin) is expected to inactivate Xgsk-3 via an intracellular signaling pathway triggered by its binding to an extracellular receptor. Although both Xwnt-8 and noggin can induce dorsal axis formation, neither is able to directly induce mesoderm (Christian et al., 1992; Smith and Harland, 1992), indicating that another factor, most likely Vg1, needs to cooperate with these factors in the induction of dorsal mesoderm (Thomsen and Melton, 1993). Complicating this...
view is the observation that high levels of Vg1 can also induce a complete dorsal axis, bypassing the need for Wnt or noggin (Thomsen and Melton, 1993). These observations can be rec-
ounced in a model in which Xgsk-3 is either directly inhibited or its effects are overridden by the various dorsalizing factors, resulting in the formation of dorsal mesoderm. In this model, ventral mesoderm is induced by a low level of Vg1 signaling (Fig. 7A). Dorsal mesoderm can be induced by a combination of low Vg1 and Wnt signals (Smith and Harland, 1991; Sokol et al., 1991) which leads to the repression of Xgsk-3, resulting in the activation of dorsal-specific genes (Fig. 7B). Vg1 signaling is also required, since neither Wnt (Smith and Harland, 1991; Sokol et al., 1991) nor the dominant-negative Xgsk-3 mutant are able to induce mesoderm. High levels of Vg1 could either override the repressive effects of Xgsk-3, and thus directly induce dorsal mesoderm, or Vg1 might inactivate Xgsk-3, leading indirectly to the induction of dorsal mesoderm (Fig. 7C). It is not yet clear which of the candidate dorsal-inducing factors are used in vivo, nor which pathway is used to antagonize the effects of Xgsk-3. Since Xwnt-8 is expressed only on the ventral side of the embryo after the early dorsalvenral patterning events, Xwnt-8 cannot be regulating Xgsk-3 in vivo. This may be accomplished by a maternal Wnt or, as discussed above, by noggin or Vg1. With the isolation and characterization of Xgsk-3, and the ability to perform biochemical studies on intracellular signaling pathways in Xenopus embryos and explants (Graves et al., 1994; LaBonne and Whitman, 1994), we hope to distinguish between these various possibilities.

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