**Sizzled: a secreted Xwnt8 antagonist expressed in the ventral marginal zone of Xenopus embryos**

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**SUMMARY**

An expression cloning screen was used to isolate a novel gene homologous to the extracellular cysteine-rich domain of frizzled receptors. The gene (which we called *sizzled* for secreted frizzled) was shown to encode a soluble secreted protein, containing a functional signal sequence but no transmembrane domains. *Sizzled* (*szl*) is capable of inhibiting Xwnt8 as assayed by (1) dose-dependent inhibition of siamois induction by Xwnt8 in animal caps, (2) rescue of embryos ventralized by Xwnt8 DNA and (3) inhibition of XmyoD expression in the marginal zone. *Szl* can dorsalize *Xenopus* embryos if expressed after the midblastula transition, strengthening the idea that zygotic expression of wnts and in particular of Xwnt8 plays a role in antagonizing dorsal signals. It also suggests that inhibiting ventralizing wnts parallels the opposition of BMPs by noggin and chordin. *Szl* expression is restricted to a narrow domain in the ventral marginal zone of gastrulating embryos. *Szl* thus encodes a secreted antagonist of wnt signaling likely involved in inhibiting Xwnt8 and XmyoD ventrally and whose restricted expression represents a new element in the molecular pattern of the ventral marginal zone.

Key words: *Xenopus*, wnt, frizzled, marginal zone, ventral patterning, dorsalization

**INTRODUCTION**

Over a dozen related genes are presently known to belong to the *wnt* family (Nusse and Varmus, 1992; Parr and McMahon, 1994). Since the initial identification of *int1* as an oncogene activated by retroviral insertion (Nusse and Varmus, 1982), wnt genes have been ascribed roles in a multitude of developmental processes, in a wide range of animals from nematodes to vertebrates. Wnt genes encode secreted proteins that have so far resisted purification in both soluble and active form, a property that has significantly delayed identification of their receptors. The recent discovery that the *Drosophila* frizzled2 gene product can directly bind wingless (wg), and mediate signaling events evoked by it, was made possible by the availability of a soluble wg preparation (Bhanot et al., 1996). *Frizzled* genes, of which almost a dozen are already known (Wang et al., 1996) encode seven-spanning transmembrane proteins, with a conserved extracellular cysteine-rich domain (CRD), which is necessary and sufficient for wg binding (Bhanot et al., 1996). The first member of the family, *Drosophila frizzled1* is a tissue polarity gene (Vinson et al., 1989) required for the proper proximodistal orientation of bristles on the body and for the mirror symmetric arrangement of ommatidia in the compound eye (Zheng et al., 1995). Before *frizzled* genes were identified as receptors for wnts, another component of the wg pathway, *dishevelled* was also known as a tissue polarity gene (Krasnow et al., 1995).

The wnt pathway is implicated in early axis induction events and mesoderm patterning in *Xenopus* (Moon, 1993; Kimelman et al., 1992). Although the involvement of specific wnt genes in axis specification remains speculative, maternal stores of a downstream component of the wnt pathway, β-catenin, are clearly required for axis formation (Heasman et al., 1994). This is likely due to β-catenin being necessary and sufficient for the formation of a dorsovegetal blastula organizer also known as the Nieuwkoop center (Wylie et al., 1996; Fagotto et al., 1997) which, in turn, induces the gastrula organizer in the overlying dorsal marginal zone (DMZ). The gastrula (or Spemann’s) organizer, also known as the dorsal marginal zone, patterns the rest of the equatorial region of the embryo, known as the ventral lateral marginal zone (VLMZ). As a result, the lateral regions of the VLMZ will form muscle and kidney while the most ventral zone will become blood and mesenchyme. The DMZ will develop intoprechordal plate and notochord. Ectopic expression of wnt genes has dramatically different effects depending on the time and place of expression. Before the mid-blastula transition (MBT) ventral wnt expression (from injected mRNA), induces dorsal structures and results in twinned embryos while post-MBT expression (from injected DNA) results in ventralized embryos (reviewed by Kimelman et al., 1992). *Xenopus* wnt8 (*Xwnt8*) is expressed zygotically in VLMZ, being excluded from the organizer (Christian et al., 1991). Xwnt8 inhibition by a dominant-negative construct decreases XmyoD expression in the VLMZ (Hoppler et al., 1996). Xwnt8 therefore normally functions to antagonize dorsal marginal zone (DMZ) signals and to positively regulate lateroventral mesoderm formation, in particular to maintain...
XmyoD expression laterally in the region fated to become somitic muscle.

We report here the expression cloning and properties of a secreted protein homologous to the CRD of frizzled genes, but which lacks any obvious transmembrane domain. This gene, which we call **sizzled** (for secreted frizzled) can antagonize Xwnt8 in a number of assays. **sizzled** is expressed in an unusual pattern, in a restricted ventral territory of the marginal zone. Its action in the ventralmost subset of Xwnt8-expressing cells presumably inhibits Xwnt8 signals ventrally. Frzb, a distantly related Xwnt8 inhibitor has recently been described and is expressed in the organizer (Leyns et al., 1997; Wang et al., 1997) in a pattern complementary to that of Xwnt8. Xwnt8 signaling thus appears to be antagonized in both dorsal and ventral marginal zones by the action of frizzled-related secreted factors, subdividing the marginal zone into at least four domains.

**MATERIALS AND METHODS**

**Xenopus embryos**

Pigmented and albino *X. laevis* embryos were obtained by in vitro fertilization, dejellied and cultured in 0.1× Marc’s Modified Ringer’s (MMR) containing 50 μg/ml gentamycin at 16-18°C. Staging of *Xenopus* embryos was done according to Nieuwkoop and Faber (Nieuwkoop and Faber, 1967). Embryos were injected in 1× MMR + 5% Ficoll and then transferred to 0.2× MMR + 5% Ficoll until further manipulations or harvesting. DNA or RNA dissolved in water were injected at a volume of 10 nl perblastomere if injections were done at the 2-4 cell stage or 1 nl per blastomere at the 32-cell stage. Animal cap explants were harvested from stage 8 embryos and cultured in 0.5× MMR + 1 mg/ml BSA until the indicated stage reached by control embryos raised at the same temperature.

**Transgenic embryos**

Transgenic *Xenopus* embryos were generated by restriction enzyme-mediated integration of linearized plasmid DNA into permeabilized sperm nuclei, as developed by Kroll and Amaya (Kroll and Amaya, 1996). Nuclear transplants were done in 0.4× MMR + 5% Ficoll and embryos were raised in 0.2× MMR + 5% Ficoll after approximately stage 7.

**Library construction and cloning**

A directional cDNA library from stages 14 through 20 *Xenopus* embryos was built in the plasmid vector pCS2+ (Turner and Weintraub, 1994). Capped RNA was synthesized by in vitro transcription from pools of 100-200 independent transformants as described (Krieg and Melton, 1984). 1-2 ng of RNA from each pool were injected into one ventral blastomere of 4-cell stage embryos. Embryos were score visually at stages 11, 14, 18 and 26 for the presence of duplicated body axis, a combination of 4-nitro blue tetrazolium (NBT) + BCIP or BM purple (Boehringer Mannheim) was used for detection. In most injection experiments, 10 pg RNA encoding a nuclear localized GFP was used as a lineage tracer which was subsequently detected in double in situ hybridization using an antisense GFP probe. Embryos were cleared in Murray’s clearing reagent; both cleared and uncleared embryos were imaged on either a Zeiss Steml stereo microscope or a Zeiss Axioskop equipped with a Sony 3 chip color video-rate CCD camera, controlled by Northern Exposure software (Phase 3 Imaging). Immunofluorescence was performed according to standard methods. The 9E10 monoclonal antibody was used to detect myc-tagged szl in stage 10.5 injected embryos using a cyanine 3-conjugated secondary antibody (Jackson ImmunoResearch). Nuclei were stained with DAPI. Confocal images were collected on a Zeiss LSM-410 confocal microscope using a 40x/1.2NA water immersion lens. Coincident Z series were obtained for both the cy3 and the DAPI signal. All images were imported into Adobe Photoshop to make composite figures. Image contrast and brightness were adjusted only to match printer output.

**RT-PCR**

RT-PCR for siamois was done as described by Brannon and Kimelman (1996). 25 cycles were used to detect the siamois cDNA. Each RT reaction contained RNA extracted from 10-20 animal cap explants or 2 whole embryos. Each PCR reaction corresponded to one animal cap explant or one tenth of a normal embryo. The qualitative nature of the RT-PCR was tested by amplifying serial dilutions of total cDNA and siamois plasmid standards.

**RESULTS**

**Identification of sizzled (szl), a secreted protein homologous to frizzled**

Small pools of RNA representing 100-200 independent clones (Lustig et al., 1996) of a *X. laevis* stage 14-20 cDNA library were screened for the ability to generate a duplicated body axis (Lemaire et al., 1995) or any other visible developmental perturbation in *Xenopus* embryos. 1-2 ng of capped synthetic RNA from each pool was injected into one ventral blastomere of 4-cell stage embryos. We screened approximately 300 pools (representing 50,000 independent clones). One of the positive pools reproducibly caused embryos to develop significantly enlarged dorsosanterior structures (most notably large cement glands). Sib selection of this pool resulted in the isolation of a single clone, as little as 10 pg of which was capable of generating the same phenotype in injected embryos. This phenotype...
was dramatically enhanced in embryos injected with increasing amounts of RNA (100 pg to 1-2 ng). These embryos (see Fig. 1B; the two left embryos have been injected with 200 pg szl RNA) appeared hyperdorsal, with large heads and cement glands (indicated by arrows in Fig. 1B and also by bottom arrow in Fig. 3B), severely stunted and kinked trunks and tails, wide and misshapen neural plates and folds which often did not close (in Fig. 1B, compare the region between arrowheads in the szl-injected versus the uninjected embryo). Part of the phenotype is reminiscent of the appearance of Xwnt5A-injected embryos (Moon et al., 1993). A histological examination of szl-injected embryos (see Fig. 1C, frontal section through such an embryo) reveals significantly expanded and hypercellular paraxial mesodermal tissue on the injected side, consistent with the presence of an enlarged muscle actin expression domain on that side (see Fig. 3A). The positive clone was sequenced and found to contain a single open reading frame encoding a protein 281 amino acids in length, with a calculated relative molecular mass of $31.8 \times 10^3$. In a sequence database search using the BLAST algorithm, the ORF was homologous to an amino-terminal portion of *frizzled* genes which corresponds to the extracellular cysteine-rich domain (CRD); however, although it contained a strong putative signal sequence, it did not contain any obvious transmembrane domain. We named this new gene *sizzled*. While this manuscript was in preparation, a distantly related gene called *frzb* was described in *Xenopus*. The comparison between szl and frzb sequences is shown in Fig. 1A. Although clearly related by sequence and function, the two genes are only 18% identical and have completely different expression patterns (see below).

**Szl is a secreted protein**

The presence of a putative signal sequence and the absence of an apparent transmembrane domain suggested that szl is secreted. We first determined whether an epitope-tagged szl (generated by attaching 6 copies of the myc epitope to the carboxy terminus) was secreted. The tag did not affect the activity of the protein, as SzlMT had the same effects as wild-type szl in embryo injections (not shown). SzlMT RNA was injected into 2-cell stage embryos and the SzlMT protein was detected by immunofluorescence in stage 10.5 embryos. Confocal micrographs (Fig. 2A and B, showing 2 focal planes of a z-series, located 15 µm apart) demonstrate that szlMT localizes to the cell surface where it tends to aggregate. Little signal can be seen inside the cell except occasional punctate staining suggesting the tagged protein efficiently reaches the cell surface.

To determine if szl can be released from cells in a soluble form, we transiently transfected 293T cells with an expression construct in which a simian CMV promoter drives szlMT expression (CS2+szlMT). We then harvested the proteins released by the cells in low-serum media. SzlMT present in the media and the cells, respectively, was analyzed by SDS-PAGE followed by western blotting with the 9E10 monoclonal. The positive clone was sequenced and found to contain a single open reading frame encoding a protein 281 amino acids in length, with a calculated relative molecular mass of $31.8 \times 10^3$. In a sequence database search using the BLAST algorithm, the ORF was homologous to an amino-terminal portion of *frizzled* genes which corresponds to the extracellular cysteine-rich domain (CRD); however, although it contained a strong putative signal sequence, it did not contain any obvious transmembrane domain. We named this new gene *sizzled*. While this manuscript was in preparation, a distantly related gene called *frzb* was described in *Xenopus*. The comparison between szl and frzb sequences is shown in Fig. 1A. Although clearly related by sequence and function, the two genes are only 18% identical and have completely different expression patterns (see below).
recognizes the myc epitope. As shown in Fig. 2C, szlMT is present in soluble form in the medium (lane 2). An estimated 5-10% of szlMT is secreted; the rest remains associated with the cellular pellet (lane 1).

These findings, which are consistent with szl sequence analysis demonstrate that szl is a secreted protein that localizes to the extracellular space.

**Szl dorsalizes embryos when expressed after the midblastula transition**

We further characterized the szl overexpression phenotype by injecting szl RNA into the 4 different tiers of blastomeres at 32-cell stage. Although the most pronounced phenotype was observed when szl was injected into tiers A and B, all injected embryos displayed the szl phenotype, consistent with szl being a secreted protein capable of acting at a distance from its source. We next investigated the effects of expressing szl after the mid-blastula transition (MBT) from a plasmid construct in which szl was driven by the simian cytomegalovirus (sCMV) early promoter/enhancer (CS2+szl). DNA-injected embryos (not shown) had the same phenotype as RNA-injected embryos, suggesting that post-MBT expression of szl is sufficient to produce the observed phenotype. Moreover, transgenic *Xenopus* embryos expressing szl under the control of the sCMV promoter/enhancer, generated by the method of Kroll and Amaya (Kroll and Amaya, 1996) showed the same phenotype (not shown).

The dorsalization effect seen after szl injections was examined in more detail at the level of both late and early mesodermal markers by double in situ hybridization to detect both the respective marker and the lineage tracer coinjected with szl at 2- or 4-cell stage. When late markers were examined at stages 24-26, the injected side of the embryos showed a strong increase in muscle actin staining (Fig. 3A, compare top embryos to the bottom one). The notochord (as seen by sonic hedgehog and collagen type II staining) was larger but also shorter and thinner than in normal embryos, with rare instances of partial duplications (Fig. 3B, embryo stained for *Xenopus* sonic hedgehog, with the arrow pointing to the secondary notochord). We next investigated how szl injections perturb early marginal zone patterning. Dorsal szl injections markedly expanded the expression domain of *gooseoid* (gsc; not shown), a homeobox gene expressed in the first involuting dorsal blastopore cells (Cho et al., 1991) and inhibited the expression of Xpo (Fig. 3C; note absence of staining on the left side, where injected szl RNA localizes), a ventroposterior gene (Sato and Sargent, 1991). Abundant ectopic expression of Xnot, a homeobox expressed mainly in the future notochord (von Dassow et al., 1993) was seen in the marginal zone overlapping with injected szl (Fig. 3E, compare injected right side with left side and with the uninjected embryo in D). In normal embryos, the region expressing Xnot elongates dramatically as gastrulation proceeds, paralleling the convergent extension movements in the prospective notochord. In szl-injected embryos, the endogenous Xnot expression fails to extend and is significantly shorter along the anteroposterior axis than in stage-matched uninjected embryos (Fig. 3D; compare to the szl-injected embryo in 3E, which is also delayed in blastopore closure). This suggests szl inhibits convergent extension and provides an explanation for the stunted appearance of szl-injected embryos. The expression of *Xenopus* brachyury in the marginal zone was unaffected by szl injections (Fig. 3F).

**Szl defines a new early ventral domain of gene expression in the marginal zone**

Though these effects of szl expression on mesodermal markers explain its ectopic effects, they tell us little about its function. We therefore examined the expression pattern of szl by reverse transcriptase PCR (RT-PCR) and whole-mount in situ hybridization (ISH). As determined by RT-PCR, szl starts to be expressed after MBT (not shown) and continues throughout gastrulation and neurulation. We did not detect the presence of maternal szl transcripts. As seen by ISH, just before gastrulation begins (stage 10) szl is diffusely expressed in the animal cap of the embryo. Early during gastrulation the expression levels increase significantly and the szl mRNA becomes restricted to the ventral marginal zone (VMZ) and ventral animal cap. By stage 10.25, szl is found in the ventral blastopore lip where it occupies a sector of approximately 120 degrees (see Fig. 4A), which becomes narrower in stage 10.5 (Fig. 4E) and 11 (Fig. 4J) embryos. Expression is highest near the blastopore lip and decreases on the ventral side toward the animal pole. At later stages, szl shows a wedge of expression in the ventralmost part of the involuting blastopore. At stages in the late teens and early twenties, szl is expressed along the belly, up to and including the heart-forming region (Fig. 4B; arrow points to szl expression in the heart-forming region), an expression pattern that persists in the latest stages examined.

**Fig. 2.** Sizzled encodes a secreted protein. (A,B) Confocal micrographs of *Xenopus* embryonic (stage 10.5) cells expressing myc-tagged sizzled. Two serial section planes located 15 μm apart are shown. The tagged protein appears red (a secondary antibody conjugated to Cy3 was used for immunofluorescent detection) while the nuclear DNA is blue (stained with DAPI). In A, the cell surfaces are roughly in the optical section plane, which corresponds to the surface of the embryo. Note the cell surface localization of szlMT in B, which shows an optical section plane 15 μm deeper into the embryo. (C) Immunoblot of myc-tagged szl secreted into the medium by 293T cells transfected with CMV-szlMT. Lane 1: cellular pellet; lane 2: tissue culture medium. Note the higher apparent MW of soluble szlMT protein in lane 2.
Response of szl to axis perturbation

We next asked how is szl expression affected by treatments that perturb the embryonic axis. Treatment of early blastulae with lithium results in dorsalized embryos. Exposure to lithium ions at the 64-cell stage completely extinguished szl expression in the vicinity of the blastopore (Fig. 4C); expression was seen and persisted throughout gastrulation only in the animal cap of lithium-treated embryos. Ventrailized embryos generated by vegetal irradiation early in the first cell cycle showed high levels of szl expression around the entire blastopore (Fig. 4D). These experiments show that szl responds to axis perturbations as expected for a gene involved in ventral patterning events.

Szl cannot rescue UV-ventralized embryos

Two genes expressed in the organizer, noggin (Smith and Harland, 1992) and chordin (Sasai et al., 1994) have dorsalizing effects on injected embryos. Both noggin and chordin also have potent UV rescuing activities and indeed, noggin was cloned based on this property (Smith and Harland, 1992). In an attempt to define the full-range of phenotypes that szl can elicit, we tested whether szl is capable of rescuing axial structures in UV ventralized embryos. 2-cell stage embryos that received a ventralizing dose of UV during the first cell cycle were injected with up to 2 ng of szl RNA (n=90, not shown). Szl had no detectable effect, all injected UV-irradiated embryos displaying the same 0 to 1 dorsoanterior index (a measure of dorsoanterior development; Kao and Elinson, 1988) as un.injected UV-ventralized embryos. UV irradiation blocks cortical rotation during the first cell cycle and embryos do not form a blastula organizer. Szl thus differs from chd and noggin by not being able to rescue dorsal development in embryos lacking an endogenous blastula organizer.

Functional antagonism between szl and Xwnt8

Since szl resembles the wnt-binding extracellular CRD of frizzled but lacks the transmembrane and intracellular domains of the receptor presumably involved in signal transduction, it follows that szl might act as an inhibitor of wnt signaling. To test this hypothesis, we looked at the interaction between szl and Xwnt8 in three different assays. We chose Xwnt8 because its expression domain contains that of szl as its ventralmost
subdomain so the two proteins can, in principle, interact during development.

To explore this potential antagonism, we first asked whether Xwnt8 can rescue the phenotype caused by szl. Szl RNA (200 pg) was injected into the 2 ventral blastomeres of 4-cell stage embryos either alone or with 100 pg of the CS2+Xwnt8 plasmid DNA which contains the Xwnt8 gene driven by the strong simian CMV promoter/enhancer. Szl RNA-injected embryos were all (n=30) short and hyperdorsal at stage 26 (see the two left embryos in Fig. 1B). When szl RNA (250 pg) was injected together with Xwnt8 DNA (100 pg) ventrally, the embryos appeared normal (n=40), demonstrating that Xwnt8 can rescue the szl overexpression phenotype (see the embryo on the right in Fig. 1B). We next tested whether szl can antagonize Xwnt8 if the two genes are expressed in two adjacent blastomeres rather than in the same one. 100 pg Xwnt8 DNA was injected into one dorsal blastomere (n=90). Some of the embryos were subsequently injected with 50 pg szl RNA into the ventral blastomere adjacent to the Xwnt8-injected blastomere (n=32) or into the same blastomere (n=30). Embryos injected dorsally with Xwnt8 DNA only were all ventralized (n=28) while szl RNA injections rescued the ventralized phenotype, whether szl was expressed in the same blastomere as Xwnt8 or in the adjacent one (not shown).

As another test of Xwnt8/szl antagonism, we explored the effects of szl overexpression on XmyoD. The expression of XmyoD in the marginal zone of Xenopus gastrulae can be inhibited by a dominant-negative Xwnt8 construct (Hoppler et al., 1996). When szl mRNA was injected into one blastomere at the 2- and 4-cell stages and then XmyoD was detected by ISH at stage 10.5-14, szl caused the disappearance or significant reduction of XmyoD on the injected side (Fig. 5D). A role for szl in regulating XmyoD expression is clear from examining the pattern of XmyoD expression during development. XmyoD is initially expressed throughout the marginal
zone and is later turned off in the dorsal lip and ventrally, persisting in the lateral marginal zone (Frank and Harland, 1991), ultimately limiting the extent of muscle formation. As seen in double ISH for XmyoD and szl, the two expression domains initially overlap but they start segregating until they are completely separated around stage 13, with szl contained in the ventral wedge from which XmyoD became excluded (Fig. 5A-C). This dynamic behavior of the two genes together with the inhibition of XmyoD by szl suggest that szl could be involved in limiting the ventral expansion of the XmyoD expression domain. Although we found XmyoD to be inhibited by ectopically expressed szl, the amount of muscle actin staining on the injected side was increased (Fig. 3A). This apparent discrepancy could be due to the fact that, although an early patterning event involving XmyoD is inhibited by szl, the later muscle differentiation process is augmented in hyperdorsal szl-injected embryos by recruitment of more ventral mesodermal tissues to become muscle. In addition, there is no simple correlation between the amounts of myoD RNA and muscle tissue: XmyoD overexpression results only in very modest muscle increase (Rupp et al., 1994) while myoD gene disruption does not reduce muscle formation (Rudnicki et al., 1993).

The homeobox gene siamois (Lemaire et al., 1995) is induced by Xwnt8 in animal cap explants (Brannon and Kimelman, 1996; Carnac et al., 1996; Fagotto et al., 1997) thus offering a more quantitative assay for wnt signaling, in a simple explant system. We coinjected Xwnt8 RNA with increasing amounts of szl RNA, cut animal cap explants at stage 8 and harvested them at stage 10.5 after culturing them in isolation, in a neutral medium. Fig. 6A shows by RT-PCR analysis that, szl was capable of inhibiting siamois expression seen in response to Xwnt8 at high but not low levels of injected szl RNA (compare lanes 6 to 5 and 7). If szl inhibits Xwnt8 by direct interaction, a component of the wnt pathway downstream of Xwnt8 such as β-catenin should not be inhibited by szl. We tested this prediction in our animal cap assay, by coinjecting equal amounts of Xwnt8 and β-catenin RNA, respectively with szl RNA and assaying siamois expression. As shown in Fig. 6B, a dose of szl RNA that was capable of inhibiting siamois expression induced by Xwnt8 did not appreciably decrease the amount of siamois transcript induced by β-catenin in animal cap explants.

**DISCUSSION**

We have used a small-pool cDNA expression cloning strategy (Lustig et al., 1996) to identify genes capable of producing visible developmental defects in Xenopus embryos. Although axis duplication is one obvious and dramatic phenotype, other subtler perturbations can be reproducibly obtained. Using this screen we have cloned sizzled, a novel gene encoding a
Szl can antagonize Xwnt8

Frizzled proteins are strong candidates for receptors of the wnt family of secreted glycoproteins. The extracellular cysteine-rich domain (CRD) of Dfz2 alone can bind wingless (wg) but does not transduce the wg signal (Bhanot et al., 1996) so szl, which shows homology to fz CRD was expected to inhibit wnt signaling by titrating the amount of wnt protein that can bind to the receptor(s). Indeed, by 3 different assays we demonstrated an antagonism between szl and Xwnt8: (1) Xwnt8 DNA can rescue embryos dorsalized by szl and conversely – szl rescues embryos ventralized by Xwnt8 DNA injections if the two genes are either expressed in the same blastomere or in adjacent ones; (2) szl inhibits the expression of XmyoD which is known to require Xwnt8 (Hoppler et al., 1996) and (3) szl can block the induction of siamois by Xwnt8 but not by β-catenin in animal cap explants. The above suggest szl can inhibit Xwnt8, perhaps by direct binding to it, in a similar manner to Xwnt8 binding to Xenopus frzb (Leyns et al., 1997; Wang et al., 1997). Xwnt8 is not yet available in soluble form to test binding to szl. We have been unable to detect szl binding to Drosophila wg.

Szl is a potent dorsalizer

Szl was cloned based on its dorsalizing activity. Both RNA and DNA injections of szl, as well as transgenic expression in Xenopus embryos, led to the same dorsalized phenotype, suggesting that post-MBT expression of szl is sufficient for dorsalization. Wnts and in particular Xwnt8 ventralize embryos if expressed after MBT (Christian et al., 1991). With Xwnt8 being expressed zygotically in the ventrolateral marginal zone (VLMZ), this implies Xwnt8 might normally be involved in inhibiting dorsal signals. Our findings that szl can inhibit Xwnt8 and dorsalize embryos post-MBT strengthens this idea of wnts playing a role in countering signals from the dorsal side of the early embryo.

Two classes of secreted proteins have been shown thus far to be capable of ventralizing Xenopus embryos: Bone Morphogenetic Proteins (BMPs) and wnts. BMP4 can be inhibited through direct binding by noggin (Zimmerman et al., 1996) and chordin (Piccolo et al., 1996). Our studies of szl show that Xwnt8, belonging to the second class of ventralizing secreted factors can be inhibited by szl, presumably by direct binding, paralleling the antagonism seen between BMP4 and noggin/chordin. The recent description of frzb, a gene distantly related to szl but expressed in the gastrula organizer (Leyns et al., 1997; Wang et al., 1997) argues that dorsalization by szl might be mimicking a process normally controlled by frzb. It also points to a caveat of misexpression experiments where the observed phenotype is in conflict with the expression pattern of a gene. Szl is, however, the first known example of a ventrally localized gene that can dorsalize embryos after the MBT.

Szl and morphogenesis

Superimposed on their dorsalized phenotype, szl-injected embryos display morphogenetic defects similar to those produced by Xwnt5A (Moon et al., 1993), a dominant-negative Xwnt8 (Hoppler et al., 1996) or a dominant-negative Xenopus dishevelled construct (Sokol, 1996). Most prominently, embryos are short and the axis is kinked. Examination of the early Xnot expression, which marks cells fated to become notochord and thus displaying the most pronounced convergent extension movements revealed a marked decrease in the anteroposterior dimension of the Xnot expressing territory. This hinted at the inhibition of convergent extension as a possible explanation for the stunted phenotype. Beta catenin, the vertebrate homologue of Drosophila armadillo and downstream of wnt and dishevelled in the wnt pathway, interacts with the cytoplasmic domain of cadherins. Cadherins are involved in calcium-mediated adhesion between cells. Wnt signaling, acting through β-catenin can modulate cell-cell adhesion (Hinck et al., 1994; Moon et al., 1993b; Peifer et al., 1993) and we speculate that inhibiting wnts could result in changes in cellular adhesion which, in turn, could perturb normal cellular intercalation and convergent extension. Whereas we do not know if szl actually operates to dampen convergent extension movements in Xenopus embryos, it should be noted that expression of szl occurs in a region where these movements are the least pronounced in the whole marginal zone. It will be of interest to explore the effect of szl and that of other inhibitors of the wnt pathway on cellular behavior in dorsal marginal zone explants undergoing convergent extension.

What is szl doing on the ventral side?

In apparent contrast to the dorsalizing activity of szl, the gene is mainly expressed in the ventral blastopore lip where it occupies a sector that becomes narrower as the blastopore closes and involutes. Additionally, szl responds to lithium and UV treatments in a manner consistent with its ventral expression. A similar situation is encountered in the case of the Anti-Dorsalizing Morphogenetic Protein (ADMP, a TGF-β family member; Moos et al., 1995), a molecule with ventralizing activity expressed in the organizer. These two examples of genes with expression patterns contrasting with their ectopic activity point to the existence of both positive and negative regulators of dorsal and ventral development, respectively. To our knowledge, szl is more restricted ventrally than any other transcript presently known, revealing a more detailed molecular pattern in the VLMZ. It will be of interest to elucidate what upstream signals control the localized expression of szl, perhaps by analyzing the promoter region of the gene in transgenic frog embryos. Xwnt8 has been strongly implicated in maintaining the expression of XmyoD in Xenopus embryos (Hoppler et al., 1996). Following the expression patterns of szl and XmyoD during and after gastrulation showed that szl and XmyoD initially overlap in the VMZ but XmyoD expression becomes gradually extinguished in the territory expressing szl and in two narrow stripes of tissue flanking this region. This dynamic expression pattern and our finding that szl can, by blocking Xwnt8, inhibit XmyoD expression, place szl as a candidate for restricting the expression of XmyoD to the lateral parts of the marginal zone which are fated to become muscle, thus refining marginal zone
pattern. A similar role is perhaps played by frzb in the dorsal marginal zone (Leys et al., 1997; Wang et al., 1997). Taken together, these two novel secreted proteins point to a regionalization of the marginal zone based on the activity level of the wnt pathway so that wnts would be fully active laterally and their activity inhibited dorsally and ventrally (see Fig. 7). This early regionalization prefigures later differentiation events, with muscle being limited to the dorsolateral domains, notochord to the extreme dorsal domain and blood and mesenchyme to the extreme ventral sector.

Until recently, the predominant view on the VLMZ, based on early organizer extirpation and grafting experiments, was that of a gastrula region undergoing default ventral development in the absence of dorsalizing signals. That ventral development is an active process was suggested by functional studies on BMP4 and Xwnt8, both broadly expressed in the VLMZ. More recently, two ventralizing homeobox genes expressed in the VLMZ, Xvent1 (Gawantka et al., 1995) and Vox1 (Schmidt et al., 1996) were proposed to act downstream of BMP4. Little is known about how the two ventralizing pathways initiated by BMP4 and Xwnt8 interact in the VLMZ of Xenopus embryos. Overexpressing BMP4 can, in fact, inhibit Xwnt8 expression (Schmidt et al., 1995, and our unpublished observations by in situ hybridization). The localization and activities of szl unveil a more complex picture of ventral patterning. Szl could define a region where Xwnt8 signaling is inhibited but the BMP4 pathway would be functional whereas laterally to the szl-expressing zone both pathways would operate. BMP4 could thus act either alone or in conjunction with Xwnt8 to pattern the VLMZ. Additional levels of refinement of this pattern could be provided by the localization and diffusibility of szl protein (both unknown at this point). We speculate that the increasingly complex pattern in the VLMZ might be indicative of a ventral organizing center (VOC), located 180 degrees from the organizer. The marginal zone could in principle be viewed as consisting, in part, of a mosaic of territories (Fig. 7) characterized by different levels of activity of the wnt and BMP pathways, generated by different local ratios of BMP4, Xwnt8 and their secreted antagonists.

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