Functional and biochemical interactions of Wnts with FrzA, a secreted Wnt antagonist

The Harvard community has made this article openly available. Please share how this access benefits you. Your story matters

Citation

Citable link
http://nrs.harvard.edu/urn-3:HUL.InstRepos:36305726

Terms of Use
This article was downloaded from Harvard University’s DASH repository, and is made available under the terms and conditions applicable to Other Posted Material, as set forth at http://nrs.harvard.edu/urn-3:HUL.InstRepos:dash.current.terms-of-use#LAA
**INTRODUCTION**

Determination of cell fates during development often depends on a cascade of inductive interactions. Reciprocal signaling between neighboring cells and participate in the determination of embryonic axes. Frizzled proteins constitute a large family of putative transmembrane receptors for Wnt signals. FrzA is a novel protein that shares sequence similarity with the extracellular domain of Frizzled. The *Xenopus* homologue of FrzA is dynamically regulated during early development. At the neurula stages, XfrzA mRNA is abundant in the somitic mesoderm, but later becomes strongly expressed in developing heart, neural crest derivatives, endoderm, otic vesicle and other sites of organogenesis. To evaluate possible biological functions of FrzA, we analyzed its effect on early *Xenopus* development. Microinjection of bovine or *Xenopus* FrzA mRNA into dorsal blastomeres resulted in a shortened body axis, suggesting a block of convergent extension movements. Consistent with this possibility, FrzA blocked elongation of ectodermal explants in response to activin, a potent mesoderm-inducing factor. FrzA inhibited induction of secondary axes by Xwnt8 and human Wnt2, but not by Xdsh, supporting the idea that FrzA interferes with Wnt signaling. Furthermore, FrzA suppressed Wnt-dependent activation of the early response genes in ectodermal explants and in the marginal zone. Finally, immunoprecipitation experiments demonstrate that FrzA binds to the soluble Wingless protein in cell culture supernatants in vitro. Our results indicate that FrzA is a naturally occurring secreted antagonist of Wnt signaling.

**SUMMARY**

Wnts are highly conserved developmental regulators that mediate inductive signaling between neighboring cells and participate in the determination of embryonic axes. Frizzled proteins constitute a large family of putative transmembrane receptors for Wnt signals. FrzA is a novel protein that shares sequence similarity with the extracellular domain of Frizzled. The *Xenopus* homologue of FrzA is dynamically regulated during early development. At the neurula stages, XfrzA mRNA is abundant in the somitic mesoderm, but later becomes strongly expressed in developing heart, neural crest derivatives, endoderm, otic vesicle and other sites of organogenesis. To evaluate possible biological functions of FrzA, we analyzed its effect on early *Xenopus* development. Microinjection of bovine or *Xenopus* FrzA mRNA into dorsal blastomeres resulted in a shortened body axis, suggesting a block of convergent extension movements. Consistent with this possibility, FrzA blocked elongation of ectodermal explants in response to activin, a potent mesoderm-inducing factor. FrzA inhibited induction of secondary axes by Xwnt8 and human Wnt2, but not by Xdsh, supporting the idea that FrzA interferes with Wnt signaling. Furthermore, FrzA suppressed Wnt-dependent activation of the early response genes in ectodermal explants and in the marginal zone. Finally, immunoprecipitation experiments demonstrate that FrzA binds to the soluble Wingless protein in cell culture supernatants in vitro. Our results indicate that FrzA is a naturally occurring secreted antagonist of Wnt signaling.

Key words: Frizzled, Dishevelled, Mesoderm, Dorsoventral axis, Morphogenetic movement, Activin, Xenopus

**INTRODUCTION**

Determination of cell fates during development often depends on a cascade of inductive interactions. Reciprocal signaling between neighboring cells and participate in the establishment of embryonic polarity and generates body patterns. One class of signaling molecules that are active throughout animal development includes Wnt family members (Parr and McMahon, 1994; Cadigan and Nusse, 1997). Wnt genes are related to the Wnt1-proto-oncogene and to *Drosophila* wingless (*wg*), and are expressed in a variety of tissues and organs. Different Wnt gene homologues are required for many developmental processes, including segmentation in *Drosophila* (reviewed by Perrimon, 1994), endoderm development in *Caenorhabditis elegans* (Rocheleau et al., 1997; Thorpe et al., 1997), limb polarity (Yang and Niswander, 1995; Riddle et al., 1995; Parr and McMahon, 1995), and kidney morphogenesis (Stark et al., 1994; Herzlinger et al., 1994). In addition, several Wnts are essential for proper development of brain and spinal cord (reviewed by Parr and McMahon, 1994). Wnt signaling may also be involved in the establishment of dorsoventral and anteroposterior axes (Sokol et al., 1991; Smith and Harland, 1991; McGrew et al., 1995; Itoh et al., 1995; Itoh and Sokol, 1997; Freudeu et al., 1997) and in the control of morphogenetic movements during gastrulation in *Xenopus* (Moon et al., 1993; Sokol, 1996).

Several components of the Wnt/Wg signal transduction pathway have been identified in *Drosophila*. Dishevelled (Dsh) and Armadillo (Arm) proteins are required for the response of *Drosophila* cells to Wg, whereas Shaggy/GSK3 kinase antagonizes the effect of Wg (Perrimon, 1994). Dsh is phosphorylated in response to Wg (Yanagawa et al., 1995) and transduces the Wg signal upstream of Arm. The same signaling pathway appears to be conserved in vertebrates, because *Xenopus* homologues of Dsh (Xdsh) and Arm (β-catenin and plakoglobin) and dominant negative forms of GSK3 (a homologue of shaggy), all mimic the ability of Wnts to induce a secondary body axis in *Xenopus* embryos (Sokol et al., 1995; Rothbacher et al., 1995; Funayama et al., 1995; Karnovsky and Klymkowsky, 1995; Dominguez et al., 1995; He et al., 1995; Pierce and Kimelman, 1995). Axin, a novel cytoplasmic product of the mouse *Fused* gene, functions as a negative regulator of the Wnt pathway (Zeng et al., 1997). Depletion of maternal β-catenin or overexpression of cadherins in *Xenopus*...
oocytes and embryos abolishes the response to a Wnt signal (Heasman et al., 1994). Consistent with a position for Dsh in the Wnt pathway, the Wnt pathway is not activated in the absence of Dsh (Heasman et al., 1994; Behrens et al., 1996; Huber et al., 1996; see Cadigan and Nusse, 1997, for a review). A similar transcription factor encoded by the fly gene pangolin, or dTCF, is required for Wg signaling in Drosophila (Brummer et al., 1997; van de Wetering et al., 1997).

Wnt signal transduction may also involve Frizzled proteins, a large family of putative receptors, which consist of an extracellular cysteine-rich domain (CRD), seven transmembrane domains, and a short cytoplasmic tail (Vinson et al., 1989; Chan et al., 1992; Wang et al., 1996; reviewed by Perrimon, 1996). Frizzled was initially identified as a Drosophila mutation that interferes with the polarized pattern of hair growth on the fly wing, often referred to as tissue polarity (reviewed by Adler, 1992). The tissue polarity is disrupted in both frizzled and dsh mutants, but not in wg mutants (Adler, 1992). Despite the lack of genetic linkage between wg and frizzled, recent studies suggest that Frizzled proteins may function as receptors for Wnts (Bhanot et al., 1996). Transfection of Dfz-2, a novel Drosophila frizzled homologue, into non-responsive S2 cell line confers responsiveness to Wg and promotes Wg binding to the cell surface (Bhanot et al., 1996). Synergistic interactions between specific Wnt and Frizzled proteins were observed upon co-injection in Xenopus embryos (He et al., 1997; Yang-Snyder et al., 1996). Together, these experiments suggest that the Frizzled proteins may function as receptors for Wnt ligands. The existence of many different Wnt and Frizzled genes, expressed during development in distinct spatial and temporal patterns, raises the question of how Wnt signaling is regulated in vivo.

In addition to Frizzled transmembrane receptors, recent studies have identified several secreted polypeptides that are related to the Frizzled extracellular domain but lack the transmembrane and intracellular domains of Frizzled (Hoang et al., 1996; Shirouzu et al., 1996; Wang et al., 1996, 1997a; Leyns et al., 1997; Mayr et al., 1997; Rattner et al., 1997; Finch et al., 1997; Salic et al., 1997; Melkonyan et al., 1997). Some of these polypeptides have been shown to antagonize Wnt signaling. We have previously isolated a bovine cDNA, encoding a protein that belongs to the same family (Duplacak, C., Xu, Q. and D’Amore, P. A., unpublished). This protein, named FrzA (Frizzled in Aorta), is secreted and is abundant in the aortic endothelium, brain and retinal neurons, lung and kidney epithelium, and cardiac myocytes. We have now isolated a Xenopus homologue of FrzA and show that it is dynamically regulated during development. To evaluate the possible role of FrzA in modulating the Wnt pathway in vivo, we overexpressed both Xenopus and bovine FrzA in the frog embryos. Wnts are known to elicit specific developmental abnormalities in Xenopus, making this system a fast and convenient model for the analysis of the Wnt pathway. To determine whether FrzA functions as a soluble Wnt-binding protein, we tested whether FrzA is capable of in vitro interactions with Wg, a Drosophila Wnt-1 homologue. Our data indicate that FrzA associates with Wnts in vitro and antagonizes Wnt signaling in the embryo.

**MATERIALS AND METHODS**

**Eggs and embryos**

Eggs were obtained by injecting Xenopus laevis females with 700 units of human chorionic gonadotropin. In vitro fertilization and embryo culture were carried out in 0.1x MMR (1x MMR =100 mM NaCl, 2 mM KCl, 1 mM MgSO4, 2 mM CaCl2, 5 mM Hepes, pH 7.6, 0.1 mM EDTA) as described (Newport and Kirschner, 1982). Embryonic stages were determined according to Nieuwkoop and Faber (1967).

**DNA constructs, RNA synthesis, microinjections and explant culture**

XfrzA cDNA was isolated by screening a Xenopus stage 17 λgt10 cDNA library at low stringency with 32P-labeled SacII-Apal DNA fragment of bovine FrzA (Duplacak et al., unpublished; GenBank accession number U85945) as described (Sambrook et al., 1989). A 1.5 kb insert from the positive phage, which contained a large open frame, was subcloned into pBSKS and sequenced in both directions. The XfrzA sequence has been submitted to the GenBank (accession number AF049908).

Capped synthetic RNAs were generated as described (Krieg and Melton, 1984) by in vitro transcription of plasmids containing the entire coding sequence of Xenopus or bovine FrzA in pXT7 (Dominguez et al., 1995), XfrzB (Leyns et al., 1997), Dsh (Sokol et al., 1995), human Wnt2 (a gift of A. McMahon), Wnt5a (Moon et al., 1993) and Wnt8 (Christian et al., 1991), using Megascript RNA transcription system (Ambion). In some experiments Xwnt8-pCS2 plasmid (a gift of R. Moon) was used for injections.

A myc-tagged form of bovine FrzA was generated by PCR with the following primers: (frzU: 5’-TCTGCGCTGGAGCGTCATCTA-3’ and frzD: 5’-AGATCTCGAGCTTGAAGACCGACTG-3’). The full coding sequence of FrzA was subcloned into plJQ3 vector that encodes a myc-epitope (Patriots et al., 1994) for transfection of COS-7 cells. HindIII and BglII-digested myc-FrzA cDNA was subcloned into pX7 for RNA microinjections. For mRNA and plasmid DNA injections, embryos were transferred to 3% Ficoll in 0.5x MMR and injected as specified in different experiments with 10 nl of RNA or DNA solution in RNase-free water.

Ectodermal explants (animal caps), constituting about one fifth of the size of the embryo, were excised at the midblastula stage (stage 8-8.5) and cultured in 0.7x MMR as described (Sokol, 1993). Total RNA was extracted from cultured explants for northern analysis, when control embryos reached stage 10.5-11.

**Whole-mount in situ hybridization and sectioning**

An antisense RNA probe was synthesized by in vitro transcription of full-length XfrzA cDNA in the presence of digoxigenin-UTP. Whole-mount in situ hybridization was performed as described (Harland, 1991). After in situ hybridization, the embryos were fixed in MEMFA (Harland, 1991), rehydrated through a graded ethanol-xylene series, embedded in Paraplast, and sectioned at 12 μm.

**Preparation of soluble Wg and FrzA proteins**

S2-Wg Drosophila cells, stably transfected with the plasmid containing wg cDNA under the control of a heat shock promoter, were used as a source of Wg protein (Van Leeuwen et al., 1994). For Wg protein preparation, S2-Wg cells were heat-shocked for 45 minutes at 37°C, cells were allowed to recover in the serum-containing medium for 35 minutes at room temperature, the medium was replaced with the serum-free M3 medium and conditioned medium was collected after 4 hours at room temperature as described (Van Leeuwen et al., 1994).
For bovine FrzA production, exponentially growing COS-7 cells were transfected with 20 μg of pJ3Wm-FrzA or pJ3Wm DNA using the calcium phosphate technique (Graham and Van Der Eb, 1973). The conditioned serum-free DMEM medium containing FrzA was collected after 3 days of culture. Conditioned media containing FrzA and Wg proteins were concentrated approximately 20 times using Centricon-30 (Amicon).

**Immunoprecipitation and western blot analysis**

Equal volume amounts of conditioned media containing soluble myc-FrzA (or control supernatant from pJ3Wm-transfected COS-7 cells) and Wg protein (or S2-cell-conditioned medium) were mixed and incubated for 10 minutes at room temperature. Myc-specific 9E10 hybridoma supernatant (15 μl) (Evan et al., 1985) was added and incubated overnight at 4°C. Immune complexes were immobilized on protein A Sepharose and washed three times with PBS. Washed beads and one fifth volume of concentrated conditioned medium before and after immunoprecipitation were subjected to SDS-polyacrylamide gel electrophoresis. Separated proteins were blotted onto Immobilon P membrane and probed with rabbit antibodies against Wg (a gift of R. Nusse) followed by goat anti-rabbit IgG secondary antibodies coupled with horseradish peroxidase (Jackson Labs). The same membrane was stripped in 7 M guanidine-HCl, 50 mM Tris-HCl, pH 8.0, 20 mM dithiotreitol, 2 mM EDTA and re-probed with 9E10 antibodies followed by goat anti-mouse secondary antibodies coupled with peroxidase. The protein bands were visualized using enhanced chemiluminescence.

**Northern blot analysis**

Total RNA was prepared from explants and embryos as described (Itoh et al., 1995). For northern analysis, RNA was separated in a 1% formaldehyde-agarose gel using standard techniques (Sambrook et al., 1989). RNA from ten animal caps or two embryos was loaded per lane unless noted otherwise. RNA was transferred to a GeneScreen nylon membrane (Dupont) with 20× SSPE and was sequentially hybridized with radiolabeled DNA or RNA probes (Sambrook et al., 1989). Antisense RNA probes were prepared by in vitro transcription (Krieg and Melton, 1984) from plasmids containing XfrzA, Xlim-1 (Taira et al., 1992), Otx2 (Pannese et al., 1995), chordin (Sasai et al., 1994), cerberus (Bouwmeester et al., 1996), PV1 (Ault et al., 1996), XMyoD (Hopwood et al., 1989), goosecoid (Blumberg et al., 1991) and fibronectin (Krieg and Melton, 1985), using [α-32P]UTP and SP6, T3 or T7 RNA polymerase. DNA probes for Xbra (Smith et al., 1991), Xnr3 (Smith et al., 1995) were radiolabeled with [α-32P]dCTP by Klenow enzyme using random hexamer primers (Sambrook et al., 1989). After hybridization, the probe was stripped by boiling in distilled water. The same membrane was used for hybridization with all probes.

**RESULTS**

**XfrzA is dynamically regulated during development**

To isolate the Xenopus homologue of FrzA, we probed a Xenopus neurula cDNA library at low stringency with bovine FrzA cDNA. The 1.5 kb insert from the positive phage contained an open reading frame encoding a protein highly similar to bovine FrzA and human FRP1 (Finch et al., 1997), but not to other members of the Frizzled family. For instance, XfrzA and XfrzB have 23% similarity at the amino acid level whereas XfrzA and FRP1 have 66% similarity. The comparison between XfrzA and other FrzB/FRP genes is shown in Fig. 1.

The expression of XfrzA during development was studied by northern blot analysis (Fig. 2). XfrzA probe detected a single mRNA species with a size of approximately 2 kb. The maternal contribution to XfrzA expression is extremely low to negligible at the blastula and early gastrula stages. The zygotic signal was significant only by the end of gastrulation and was well pronounced at the neurula, tailbud and tadpole stages. There was a reproducible decline in XfrzA mRNA levels at stage 22, which is consistent with in situ hybridization data.

Whole-mount in situ hybridization analysis revealed spatial aspects of XfrzA expression. XfrzA transcripts are not detected in gastrulae (Fig. 3A), and the transcripts are first seen in somitic mesoderm at the neurula stage (Fig. 3B,C,G). At the tailbud stages, XfrzA is found in neural crest cells, heart, otic vesicle, pronephros, endoderm and the ventral part of the neural tube at the hindbrain level (Fig. 3E,H-M). Interestingly, heart-specific expression of XfrzA was confined to the myocardium with little or no staining in the pericardium or endocardium areas (Fig. 3L,K).
The effect of FrzA mRNA on early development

Since FrzA is similar to the extracellular domain of Frizzled, it may modulate Wnt function in vivo. To analyze the potential biological role of FrzA, we overexpressed FrzA in Xenopus embryos by microinjection of its mRNA. Ventral injections of 0.5-1 ng of FrzA mRNA in four-cell embryos had no significant effect on the overall morphology of developing tadpoles (Fig. 4A). Occasionally, the tail of injected embryos was slightly bent. We suspect that in these cases the injected mRNA may have been mislocalized to a lateral position. Bovine or Xenopus FrzA mRNA did not induce secondary axes on the ventral side, indicating that neither stimulates the Wnt signal transduction pathway.

Dorsal injections of 1 ng of bovine FrzA mRNA reproducibly led to dorsally curved embryos with severely truncated posterior tissues (Fig. 4B). Head development was not significantly affected in these embryos. The lack of axis extension was seen in a majority of injected embryos and was observed using both myc-tagged and non-tagged variants of FrzA. This phenotype is very similar to inhibition of axis extension that occurs in embryos when Wnt signaling is blocked by overexpression of Xdd1, a dominant negative form of Xdsh (Sokol, 1996). Microinjections of XfrzA resulted in a similar phenotype (data not shown), whereas control dorsal injections of the same dose of EF-1a mRNA did not alter normal development. These observations suggested that FrzA is a naturally occurring inhibitor of Wnt signaling.

Since Xdd1 was reported to inhibit morphogenetic movements of ectodermal explants (animal caps) stimulated with mesoderm-inducing factors (Sokol, 1996), we assessed whether FrzA has the same activity on cell movements. Animal caps were isolated from FrzA mRNA-injected embryos at midblastula stage and were treated with activin, a potent mesoderm-inducing factor. Strong inhibition of animal cap elongation was observed in the presence of FrzA mRNA when compared with uninjected controls (Fig. 5). The effect was dose-dependent, as lower doses of FrzA mRNA (0.1-0.2 ng) caused less pronounced inhibition. FrzA did not interfere with the reception and transduction of activin signals, because several early marginal zone gene markers, including Gsc (Blumberg et al., 1991), Xlim-1 (Taira et al., 1992) and chordin (Sasai et al., 1994) were activated in response to activin in the presence of FrzA (data not shown). Taken together, the developmental effects of FrzA suggest its interference with Wnt signaling in the embryo (see also Hoppler et al., 1996).

Fig. 2. Expression of XfrzA during Xenopus development. Total RNA was extracted from different stages of Xenopus embryos as indicated, and probed with [$\alpha$-32P]UTP-labeled antisense XfrzA RNA. Two embryo equivalents were loaded per lane, except that only one embryo equivalent was loaded for stage 22, and 3/4 equivalents for stage 35. Fibronectin (FN) was used as a loading control.

Fig. 3. Localization of XfrzA mRNA in Xenopus embryos by whole-mount in situ hybridization (A-F) and sections (G-M). XfrzA antisense probe was used in A-C and E; control En-2 antisense probe was used in D and F. (A) Stage 10.5; (B-D,G) stage 17; B is dorsal and C is lateral view. (E,F,H,J,K,M) Stage 29-30. (I,L) Stage 35. (B-F) Anterior is to the left, s, somitic tissue; ov, otic vesicle; nc, neural crest cells; nt, neural tube; not, notochord; bv, brain ventricle; nr, neural retina; pn, pronephros; hb, hindbrain; mc, myocardium; pc, pericardium; ec, endocardium; h, heart anlage; en, endoderm.
FrzA blocks axis-inducing activities of Xwnt8, but not that of *Xenopus* Dishevelled

To test whether FrzA functions in the embryo by preventing the interactions of Wnt ligands with the proper receptor, we evaluated whether it suppresses the axis-inducing activity of Xwnt8. In addition, if FrzA acts outside of the cell, then we would expect that it should not interfere with intracellular mediators of Wnt signaling such as Dishevelled (Dsh). Both Xwnt8 and Xdsh mRNAs are known to induce a complete secondary axis upon microinjection in a ventral blastomere (Sokol et al., 1991, 1995). Co-injection of Xwnt8 mRNA and bovine or *Xenopus* FrzA mRNA in a single ventral blastomere resulted in a complete inhibition of secondary axis induction by Xwnt8 (Table 1, Fig. 6A,B). In contrast, FrzA mRNA failed to inhibit axis induction by Xdsh mRNA (Table 1, Fig. 6C, D), a result that is consistent with the position of Dsh downstream of Frizzled in the Wnt signal transduction pathway. Even at the twofold lower doses of Xdsh mRNA, at which only partial axes are induced (Sokol et al., 1995), we failed to detect significant inhibition by FrzA (data not shown).

The effects of FrzA on early marginal zone markers

Morphological observations were further confirmed by the molecular data showing that Xwnt8-dependent activation of two marginal zone markers Xnr-3 and chordin was inhibited by FrzA in ectodermal explants (Fig. 7A). Expression of Otx2 was not blocked to the same degree, suggesting that it may be activated by a different mechanism. On its own, FrzA did not stimulate any tested marginal zone markers in animal cap explants (Fig. 7A). In contrast, GSK3, another inhibitor of the Wnt pathway, upregulates Otx2 expression in animal caps (Itoh et al., 1995). These observations indicate that the pathway is likely to branch out or to cross-talk with different signal transduction pathways.

We then compared the effects of FrzA and FrzB mRNAs on the expression of several marginal zone markers. Wnt2 expression in ventral vegetal cells leads to induction of Chd,
Otx2, Cer and XmyoD (Fig. 7B). This induction was blocked by injection of Wnt2 mRNA with FrzA or FrzB, consistent with the effect of XfrzA on animal cap explants.

Injection of FrzA RNA into the dorsal margin led to suppression of XmyoD, and a weak increase in expression of Otx2 (Fig. 7B, XFA/d, and data not shown). Both XfrzB and a dominant negative Wnt construct have been reported to block XmyoD expression (Leyns et al., 1997; Wang et al., 1997a; Hoppler et al., 1996). While XfrzB was shown to cause an increase in chordin (Leyns et al., 1997) and to induce a partial secondary axis (Wang et al., 1997a), we detected no other significant changes of dorsal markers in embryos overexpressing XfrzA (Fig. 7B and data not shown). Together, these results argue that FrzA is a direct inhibitor of Wnt signaling and may block the accessibility of Wnts to their endogenous receptors. Our findings lend further support to participation of some Wnt ligand(s) in both myogenesis and head development, since MyoD and Otx2 are known to be involved in these processes.

Selectivity of FrzA-Wnt interactions

To determine if FrzA can modulate the function of other Wnt products, we tested whether it interferes with the activity of Wnt2. When mRNA encoding human Wnt2 (a gift of A. McMahon) was injected in a ventral blastomere, it triggered the formation of complete secondary axes (Table 1), similar to those induced by Xwnt8, Wnt1 (Sokol et al., 1991) and Wnt3a (Wolda and Moon, 1993). Coinjection of bovine FrzA mRNA inhibited this axis-inducing activity of human Wnt2, indicating that FrzA can interact with both Wnt ligands (Table 1). However, we did not detect a significant effect of FrzA on activity of Xwnt8 DNA (Christian and Moon, 1993) or BMP-4 RNA (Dale et al., 1992; Sasai et al., 1995), which are both known to ventralize early embryos. For Xwnt8 DNA experiments, 10, 20 and 40 pg of Xwnt8-pCS2 plasmid were injected into both dorsal blastomeres of four-cell stage embryos in the absence or presence of 0.1-0.4 ng of XfrzA RNA. At 40 pg, Xwnt8 DNA strongly inhibited anterior brain development, whereas 10 pg only caused a marginal effect. Although XfrzA RNA had a pronounced effect on convergent extension movements, it failed to restore forebrain development at any dose of injected plasmid.

A distinct group of Wnts, including Xwnt5a, Xwnt11 and Xwnt4, have developmental effects that differ from the axis-inducing activity of Xwnt8 (Moon et al., 1993; Du et al., 1995). These Wnts fail to induce a complete secondary axis, but inhibit morphogenetic movements in the embryo. To determine whether FrzA exhibits any selectivity with respect to its interactions with Wnts, we co-injected different amounts of
Xwnt5a mRNA and 1 ng of FrzA mRNA into the dorsal marginal region of four-cell embryos. Overexpression of Xwnt5a suppressed morphogenetic movements in 48 out of 62 injected embryos as described previously (Moon et al., 1993). The phenotype resulting from disrupted morphogenetic movements is not easily quantified, and we failed to detect a significant modulation of the Xwnt5a activity by FrzA (axis extension was inhibited in 67 out of 71 injected embryos). These experiments suggest that FrzA may interact with only a subset of Wnt products.

**FrzA is actively secreted and associates with Wg in vitro**

In order to determine whether FrzA is secreted, we analyzed the production of FrzA by COS-7 cells transfected with a cDNA encoding myc-tagged bovine FrzA. After 3 days of culture, large amounts of FrzA protein were present in the conditioned medium (Fig. 8A), whereas the protein was virtually undetectable in the lysates of the transfected cells. These observations indicate that FrzA is an actively secreted protein.

We next wanted to test whether FrzA is able to bind Wnts. Because the majority of Wnts cannot be easily obtained as functionally active soluble proteins, we utilized culture supernatants of the S2-Wg cell line, which has been stably transfected with wg cDNA under the control of a heat-shock promoter (van Leeuwen et al., 1994). In contrast to FrzA, Wg was mainly present in cell lysates (data not shown), with only a small proportion detected in the conditioned medium (Fig. 8B). Concentrated culture media containing Wg and myc-FrzA products were mixed in equal volumes, and myc-FrzA protein was precipitated with myc-specific 9E10 monoclonal antibodies. Subsequent western analysis with anti-Wg antibodies revealed the presence of Wg in the immune complexes (Fig. 8B,C, top panels). 9E10 antibodies reacted specifically with myc-FrzA and did not precipitate Wg from Wg-containing medium. The efficiency of myc-FrzA immunoprecipitation was verified by stripping the membrane and reprobing it with 9E10 antibodies (Fig. 8B,C, bottom panels). Conditioned media containing Wg and FrzA proteins revealed that FrzA was substantially depleted with 9E10 antibodies, but that the amount of Wg protein in the media did not significantly change (Fig. 8C). These results indicate that while Wg associates with FrzA in vitro, only a small portion of Wg is present in complex with FrzA.

**DISCUSSION**

This study evaluates a potential role of a novel protein (FrzA), which is highly homologous to the extracellular domain of Frizzled receptors, in Wnt signal transduction. We demonstrate that FrzA is actively secreted into the culture medium, binds...
Wg in vitro and inhibits the activities of two Wnt products, Xwnt8 and human Wnt2 in the embryo. Developmental effects of FrzA are similar to the effect of a dominant negative Xdsh (Sokol, 1996), and are likely to reflect inhibition of Wnt signaling in the embryo.

Although recent reports have shown that Wg binds to the surface of cells transfected with Frizzled cDNAs (Bhanot et al., 1996), and that FrzB product binds to cells transfected with a membrane-bound form of Wnt-1 (Leyns et al., 1997), these findings do not demonstrate biochemical association of the two proteins. XfrzB was reported to associate with in vitro translated Xwnt8 product (Wang et al., 1997a), but the functional activity of the in vitro translated Xwnt8 was not demonstrated (Wang et al., 1997). Our data extend these observations by demonstrating that a soluble Wnt ligand (Wg), which is secreted by cells in a biologically active form (Van Leeuwen et al., 1994), forms a biochemical complex with a secreted Frizzled-related protein (Fig. 8).

Other proteins related to the Frizzled CRD were identified recently (Shirozu et al., 1996; Rattner et al., 1997; Finch et al., 1997; Salic et al., 1997; Mellkonyan et al., 1997), and two of them, SARP2 (Mellkonyan et al., 1997) and FRP1 (Finch et al., 1997), are highly related to FrzA (Fig. 1). Among the best characterized Frizzled CRD-related proteins are FrzB homologues (Hoang et al., 1996; Mayr et al., 1997; Leyns et al., 1997; Wang et al., 1997a). FrzB transcripts are abundant in bovine cartilage (Hoang et al., 1996), mouse central and peripheral nervous system, and in the kidney (Mayr et al., 1997). In contrast, FrzA is found in adult cow brain, heart, kidney, spleen, lung and in some blood vessels (Djuala et al., unpublished). In the early gastrulae, XfrzB transcripts are restricted to the organizer region (Leyns et al., 1997; Wang et al., 1997); however, XfrzA mRNA is not detectable at this time of development (Fig. 3). At tailbud stages, FrzB expression is confined to the stomodeal-hypophyseal anlage (Leyns et al., 1997; Wang et al., 1997), whereas FrzA is strongly expressed in the developing myocardium, otic vesicle, endoderm, pronephros and neural crest (Fig. 3). Thus, tissue distribution of FrzA and FrzB in Xenopus embryos suggest that these molecules may locally control Wnt activities, and that the specificity of their effects could be primarily determined by their expression patterns. The existence of multiple Wnt ligands and their putative receptors and CRD-related proteins raises the question of whether there is any selectivity in these ligand-receptor interactions. To account for the observed synergy of Xwnt-5a and Hfz-5 in axis induction, Hfz-5 was proposed to function as a receptor for Xwnt-5a (He et al., 1997). With regards to soluble Frizzled CRD-related proteins, only FrzB has been reported to interact with Xwnt8 and Wnt1, but not with Wnt-3A, -5A or -11 (Wang et al., 1997a,b; Leyns et al., 1997). Our experiments suggest that FrzA may interfere with the activities of at least three different Wnts, including Xwnt8, Wnt2a and Wg, yet we failed to detect an effect of FrzA on the ability of Xwnt5a to block morphogenetic movements. FRP-1 inhibits functional activity of Xwnt8, but has only a weak effect on Xwnt-3a (He et al., 1997). While SARP-2 negatively regulates intracellular concentration of β-catenin, SARP-1 leads to its upregulation, suggesting a stimulatory effect on the Wnt pathway (Mellkonyan et al., 1997). Together, these observations suggest that different Frizzled CRD-related proteins may have diverse effects and different specificities in vivo. Further analysis requires purified preparations of various Wnts, which are not currently available.

Soluble binding proteins have been described for a number of different ligands. For example, a soluble receptor for vascular endothelial growth factor (VEGF) was shown to bind to the ligand and inhibit its biological activity (Kendall and Thomas, 1993). Soluble forms of the fibroblast growth factor (FGF) receptor have been identified in blood and are speculated to control FGF activities (Hanneken et al., 1994). The existence of membrane-anchored and soluble forms of growth factors and their receptors may reflect a common mechanism of controlling short- and long-range intercellular communications and establishing tissue boundaries in the embryo.

We thank R. Nusse for the S2-Wg cell line and for anti-Wg antibodies, J. Chernoff for p33Q2R, R. Moon for Xwnt5a and Xwnt8, A. McMahon for human Wnt2, E. De Robertis for chordin, BMP-4 and XfrzB, R. Harland for Xnr3, M. Jamrich for PV1, J. Gurdon for XMyoD. We also thank K. Itoh and V. Krunik for comments on the manuscript. This work was supported by the grants from the March of Dimes Birth Defects Foundation and NIH (HD31247) to S. Sokol and by the NIH grants EY35518 and CA45458 to P. A. D’Amore.

REFERENCES


Du, S. J., Purcell, S. M., Christian, J. L., McGrew, L. L. and Moon, R. T.


