Cloning and Expression of the Wnt Antagonists Sfrp-2 and Frzb during Chick Development

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The Wnt genes are known to play fundamental roles during patterning and development of a number of embryonic structures. Receptors for Wnts are members of the Frizzled family of proteins containing a cysteine-rich domain (CRD) that binds the Wnt protein. Recently several secreted frizzled-related proteins (Sfrps) that also contain a CRD have been identified and some of these can both bind and antagonise Wnt proteins. In this paper we report the expression patterns of the chick homologues of Frzb, a known Wnt antagonist, and Sfrp-2. Both genes are expressed in areas where Wnts are known to play a role in development, including the neural tube, myotome, cartilage, and sites of epithelial–mesenchymal interactions. Initially, Sfrp-2 and Frzb are expressed in overlapping areas in the neural plate and neural tube, whereas later, they have distinct patterns. In particular Sfrp-2 is associated with myogenesis while Frzb is associated with chondrogenesis, suggesting that they play different roles during development. Finally, we have used the early Xenopus embryo as an in vivo assay to show that Sfrp-2, like Frzb, is a Wnt antagonist. These results suggest that Sfrp-2 and Frzb may function in the developing embryo by modulating Wnt signalling.

Key Words: Sfrp-2 (SDF-5); Frzb; Wnt antagonist; neural; limb; heart; craniofacial; placodes; myogenesis; chondrogenesis.

INTRODUCTION

The Wnt gene family consists of at least 16 secreted glycoproteins that play fundamental roles during patterning and morphogenesis of a number of embryonic structures (reviewed by Parr and McMahon, 1994; Wodarz and Nusse, 1998). For example, Wnt signalling patterns the dorsoventral axis of the early Xenopus embryo (McMahon and Moon, 1989; Moon et al., 1997) and of the developing limb bud in mice and chicks (Kengaku et al., 1998; Parr and McMahon, 1995; Riddle et al., 1995; Yang and Niswander, 1995). During somite development, Wnt signalling from the neural tube controls formation of the medial dermamyotome (Münsterberg et al., 1995; Ikeya and Takada, 1998). In the kidney, Wnt-4 is required for the mesenchymal to epithelial transformation of the metanephrogenic mesenchyme to form the epithelial nephrons (Stark et al., 1994), whilst during skeletal development, Wnt-1, Wnt-5a, and Wnt-7a can inhibit chondrogenesis (Rudnicki and Brown, 1997; Kawakami et al., 1999). Functional studies in developing
mouse, chick, and Xenopus embryos, or in cell lines, have shown that Wnt signalling can control several cellular processes, including proliferation, cell transformation/fate, and cell adhesion (reviewed by Wodarz and Nusse, 1998).

Using functional assays the Wnt family has been subdivided into at least two subclasses. In Xenopus, Wnt-1, Wnt-3a, and Wnt-8 are able to induce axis duplication in embryos, whereas Wnt-4, Wnt-5a, and Wnt-11 block activin-induced morphogenetic movements in blastula caps but cannot duplicate the Xenopus axis (Du et al., 1995). In zebrafish embryos, signalling by Wnt-5a, but not Wnt-8, induces an increase in intracellular calcium (Siusarski et al., 1997a,b). The Wnt gene family has also been subdivided into those that can transform the C57MG mammary epithelial cell line (Wnt-1, Wnt-3a, and Wnt-7a), those that cannot (Wnt-4 and Wnt-5a), and those that can only weakly transform C57MG cells (Wnt-2, Wnt-5b, and Wnt-7b; Wong et al., 1994).

The frizzled family consists of at least 11 members in vertebrates (Wang et al., 1996), some of which have been shown to function as Wnt receptors. They are integral membrane proteins containing an N-terminal extracellular cysteine-rich domain with 10 cysteines, known as the CRD or frizzled domain, and seven putative transmembrane segments together with an intracellular domain. CRDs, which can directly bind Wnt proteins (Bhanot et al., 1996; Lin et al., 1997; Wang et al., 1997; Lercher et al., 1998; Bafico et al., 1999), have also been identified in another group of genes known as the secreted frizzled-related proteins (Sfrps). They include Sfrp-1 (also known as hFRP, FrzA, and SARP2), Sfrp-2 (also called SARPI and SDF-5), Frzb (Sfrp-3 and Fritz), Sfrp-4, SARP3, sizzled, and crescent, which have so far been characterised in humans, mice, chick, and Xenopus (Shirozu et al., 1996; Finch et al., 1997; Mayr et al., 1997; Melkonyan et al., 1997; Pfeffer et al., 1997; Rattner et al., 1997; Salic et al., 1997; Hoang et al., 1998; Lercher et al., 1998). In addition to the CRD, Sfrps also contain a novel C-terminus that lacks both the transmembrane and the intracellular sequences of the frizzled gene family. Functional studies in Xenopus have shown that Frzb, Sfrp-1, and sizzled can antagonise signalling by specific Wnt proteins. Sizzled blocks Wnt-8 signalling: FrzB inhibits Wnt-1 and Wnt-8, but not Wnt-3a, Wnt-5a, and Wnt-11; whilst Xsfrp-1 (FrzA) inhibits Wnt-1, Wnt-2, Wnt-8, and to a lesser extent Wnt-3a, but does not block signalling by Wnt-5a (Leyns et al., 1997; Mayr et al., 1997; Salic et al., 1997; Wang et al., 1997; Xu et al., 1998). The ability of the other Sfrps to inhibit Wnt signalling has not yet been reported. However, together with cerberus, dickkopf-1, and the recently identified WIF-1 protein, which are all secreted and can antagonise Wnt signalling, they form one group of proteins that may be critical in modulating Wnt function (Glinka et al., 1998; Hsieh et al., 1999; Piccolo et al., 1999).

Sfrp-1, Sfrp-2, Sfrp-4, and Frzb are expressed in a number of regions where they may modify Wnt signalling in developing mouse and human embryos (Finch et al., 1997; Hoang et al., 1996, 1998; Rattner et al., 1997; Lercher et al., 1998; Leimer et al., 1998). In the chick, only the expression of crescent, which is found in the anterior endoderm during gastrulation, has been reported (Pfeffer et al., 1997). In this paper we describe the cloning and expression of two other Sfrp family members, Sfrp-2 and Frzb, during chick development. We compare their expression during neurogenesis; during formation of the limb bud, somites, and heart; and in the development of the face. Finally, we show that chick Frzb and chick Sfrp-2 hyperdorsalise injected Xenopus embryos and block the dorsalisising activity of Xwnt-8 in ventral marginal zone explants. These results suggest that cFrzb and cSfrp-2 are antagonists of Wnt proteins.

**MATERIALS AND METHODS**

**Chick Embryos**

Fertilised Ross White chicken eggs were supplied by Poyndon Farm (Goffs Oak, Hertfordshire, UK) and were incubated at 38 ± 1°C in humidified incubators. Embryos were staged according to Hamburger and Hamilton (1951).

**Cloning of Sfrp-2 and Frzb**

Homologues of Sfrp-2 and Frzb were obtained by screening a stage 12 chick Lambda ZAP II cDNA library under low-stringency conditions as described by Francis et al. (1994). 32P-Labelled random-primed DNA probes were made from fragments encoding amino acids 49 to 242 of mouse Sfrp-2 and the full-length bovine Frzb coding sequence (Hoang et al., 1996). The mouse Sfrp-2 fragment was obtained by PsiI digestion of IMAGE clone 536389 from the EST database (Lennon et al., 1996). From 10⁴ plaques, 28 Sfrp-2 positives and 19 Frzb positives were identified in the primary screen, and 10 and 13 clones, respectively, were purified to completion under the same conditions. The Bluescript plasmids encoding the Sfrp-2 and Frzb homologues were excised following standard procedures (Stratagene). Following restriction analysis, deoxy sequencing of both strands was carried out on three independent Frzb cDNAs and one Sfrp-2 cDNA with Sequenase version 2 or a T7 kit (Amersham Pharmacia Biotech) using denatured double-stranded DNA templates. Nucleotide sequences were assembled using the Assemblylink computer program (Oxford Molecular) and the inferred protein sequences were compared with known sequences from GenBank. The DNA sequences have been deposited in the GenBank database.

**RNA Studies**

Total RNA was extracted from stage 28 embryos using Trizol (Gibco Life Technologies) and poly(A)⁺ RNA was subsequently isolated using a PolyA Tract mRNA Isolation system IV (Promega) according to the manufacturer’s instructions. Two micrograms of poly(A)⁺ RNA were denatured with 1.2 M glyoxal, 53% DMSO, and 0.01 M sodium phosphate (pH 7) for 1 h at 50°C before size fractionating on a 1.2% agarose/0.01 M sodium phosphate (pH 7) gel. The RNA was then transferred onto a Hybond-N membrane (Amersham Pharmacia Biotech) and hybridised overnight with radiolabelled probe and washed as described by Francis et al. (1994).
DNA Constructs
The Sfrp-2 antisense probe was generated by linearising the full-length clone with HindIII and transcribing with T3 RNA polymerase. Sense Sfrp-2 transcripts were generated with T7 RNA polymerase using Smal-linearised Sfrp-2 DNA as a template. The PstI fragment encoding nucleotides 667 to 1859 of Frzb was subcloned into pBluescript SK (\stretch{0.3cm}). This region encodes part of the 3' region of the Frzb protein (from amino acid 207) and 850 bp of the 3' UTR. Antisense probes for in situ hybridisation and Northern analyses of Frzb were synthesised with T3 RNA polymerase using Xhol-digested plasmid. Sense control probes were obtained with T7 polymerase using Xbal-digested plasmid. Full-length chick Sfrp-2 and Frzb were subcloned into the CS2' vector (Rupp et al., 1994) and RNA was transcribed with SP6 RNA polymerase using NotI-linearised plasmid as a template. Sox9 (Kent et al., 1996), Slug (Nieto et al., 1994), Wnt-1 (Hollyday et al., 1995), and Wnt-8c (Hume and Dodd, 1993) were used as previously described.

In Situ Hybridisation to Whole Mounts and Tissue Sections
Embryos were fixed in 4% (w/v) paraformaldehyde and processed through a methanol series for whole-mount in situ hybridisation as described by Francis-West et al. (1994) for older embryos (stages 17 to 28) and by Myat et al. (1996) for younger embryos. Alternatively, after fixation embryos were processed into wax for radioactive in situ hybridisation as described by Barlow and Francis-West (1997).

Xenopus Experiments
Xenopus embryos were obtained by artificial fertilisation as described (Dale and Slack, 1987). The embryos were dejellied in 2% cysteine hydrochloride (pH 8.1) and staged according to Nieuwkoop and Faber (1967). They were then transferred into 10% normal amphibian medium (NAM; 110 mM NaCl, 2 mM KCl, 1 mM Ca(NO3)2, 1 mM MgSO4, 0.1 mM Na2EDTA, 1 mM NaHCO3, 30 mM Hepes buffer, pH 7.5) supplemented with 4% Ficoll. For the whole-embryo phenotype experiments one ventral blastomere was injected at the four-cell stage with 1 ng of either Sfrp-2 or Frzb mRNA and embryos were cultured until stage 36 when hydorosalised embryos were scored using the dorsoanterior index of Kao and Faber (1967). They were then dejellied in 2% Hepes buffer, pH 7.5) and staged according to Nieuwkoop and Faber (1967).

RESULTS
Isolation and Sequence of Sfrp-2 and Frzb
Chick Sfrp-2 and Frzb cDNA clones were isolated from a stage 12 cDNA library using cDNA fragments encoding the mouse and the bovine homologues, respectively. Ten Sfrp-2 and 13 Frzb clones were purified and were characterised by restriction mapping. Sequence analysis of the longest cDNAs showed that chick Sfrp-2 and Frzb encode proteins consisting of 292 and 315 amino acids, respectively. Both contain the 10 conserved cysteine residues characteristic of the CRD found in other Sfrps and members of the frizzled family. Comparison of the inferred amino acid sequence with the homologues in other species showed that Sfrp-2 shares 80% identity with the mouse homologue whilst Frzb shares an average of 78% identity with the human, mouse, bovine, and Xenopus homologues. The predicted amino acid sequences of Sfrp-2 and Frzb, together with comparison with the other homologues, are shown in Figs. 1A and 1B, respectively.

Analysis of the inferred amino acid sequences using the software PrositeScan revealed a putative N-glycosylation site at asparagine 41 and a putative O-glycosylation site at threonine 147 in Frzb although none were identified in Sfrp-2. In addition, several putative hyaluronan binding sites and a region of homology termed the FUN (Frzb/Uncl-6/netrin) domain, which has similarity to a region in the
netrin-2 and Unc-6 proteins involved in axonal guidance, were identified (Fig. 1C; Serafini et al., 1994; Yang et al., 1994; Leyns et al., 1997). However, in our preliminary studies, we have not been able to demonstrate functional hyaluronan binding sites in recombinant Frzb as determined by its ability to bind to biotinylated hyaluronan.

**FIG. 1.** Amino acid alignment of (A) Sfrp-2 homologues in chicken, mouse, and human (Melkonyan et al., 1997; Rattner et al., 1997) and (B) Frzb homologues in chicken, mouse, human, cow, and Xenopus (Hoang et al., 1996; Leyns et al., 1997; Wang et al., 1997). The CRD is underlined and the conserved cysteines within this domain are shown in bold. Amino acids identical to those in chicken are indicated by dashes. Gaps (represented by dots) have been introduced to optimise the alignment. An asterisk indicates the end of the known human Sfrp-2 sequence. The predicted signal sequence cleavage site of Frzb is APA-GR and Sfrp-2 is STA-GL as described by Nielsen et al. (1997). A dagger indicates the putative N-glycosylation site at asparagine 41 and the putative O-glycosylation site at threonine 147 in Frzb. Alignment of the FUN domain of chicken netrin-2, Caenorhabditis elegans Unc-6, chicken Sfrp-2, and chicken Frzb. Residues shown in bold are conserved between all four proteins. Dots indicate gaps to increase the alignment. The sequences in (A, B, and C) were aligned using the software ClustalW 1.7.
The sizes of the transcripts are marked in kilobases.

FIG. 2. Northern analysis of Sfrp-2 and Frzb mRNAs. Two micrograms of poly(A)^+ RNA from stage 28 embryos was loaded on each lane and hybridised for probes specific for chicken (A) Sfrp-2 and (B) Frzb. The sizes of the transcripts are marked in kilobases.

Northern Analysis

The Sfrp-2 and Frzb mRNAs were analysed by Northern blotting of poly(A)^+ RNA from stage 28 embryos. Three RNA transcripts of 1.4, 2.1, and 2.6 kb, the predominant one being 2.1 kb, hybridised to both full-length and 3' UTR probes for Sfrp-2, whilst a single RNA transcript of approximately 2.3 kb hybridised to the Frzb probe (Fig. 2). The same pattern of hybridisation with both the full-length and the 3' UTR Sfrp-2 probes suggests that the three transcripts may be due to alternative polyadenylation sites, alternative splicing, or promoter usage in a single gene.

Expression of Sfrp-2 and Frzb during Chick Development

To investigate the expression of Sfrp-2 and Frzb during embryogenesis, in situ hybridisation was performed on whole embryos. This showed that Sfrp-2 and Frzb are expressed in developing neural structures and some of its derivatives, as well as the heart, branchial arches, and limb buds (Figs. 3–7). In early development Sfrp-2 and Frzb have similar domains of expression, whereas in older embryos the domains of expression are distinct. In some regions, expression of Sfrp-2 and Frzb appears to be associated with myogenesis and chondrogenesis, respectively.

Early Development

At stage 7, both Sfrp-2 and Frzb are expressed in the neural plate with lower expression in the midline (Figs. 3A, 3B, 3C, and 3E). Both genes are expressed in a graded manner, with higher levels anteriorly (Figs. 3B and 3C). At this stage, Sfrp-2 and Frzb expression encompasses Wnt-1 expression in the neural plate (data not shown) and Wnt-8c in the presumptive rhombomere 4 and posterior neural plate (Fig. 3D). In addition, Wnt-8c transcripts are present in the primitive streak, Hensen’s node, and the presumptive mesoderm (Fig. 3D and also see Hume and Dodd, 1993). By stage 9, the expression of Sfrp-2 and Frzb is distinct. At this stage, expression of Sfrp-2 is throughout the developing neural tube, with lower levels in the future posterior mesencephalon and rhombomere 3 (Fig. 3I). In anterior regions, expression is stronger in the dorsal neural tube, whereas more posteriorly, Sfrp-2 is expressed throughout the closing neural tube (Figs. 3F and 3H). In contrast, Frzb is restricted to the anterior neural tube but, as with Sfrp-2, its expression is stronger dorsally (Figs. 3G, 3J, and 3L). In addition, Sfrp-2 expression is also detectable in the surface ectoderm flanking the neural tube (Figs. 3F and 3H), whilst Frzb is found in the neural crest (Fig. 3G). There is a similar relationship between Wnt-8c and Sfrp-2 with their expression overlapping in the posterior neural tube and presumptive hindbrain, whereas at this stage, Frzb expression overlaps only with Wnt-8c in the latter region (data not shown). However, the relationship between Wnt-1, Frzb, and Sfrp-2 is more complex. In the developing brain, Frzb and Wnt-1 transcripts overlap (Figs. 3J and 3K), whereas Sfrp-2 and Wnt-1 expressions are almost complementary (Figs. 3I and 3K). Thus, Sfrp-2 transcripts but not those of Wnt-1 are found in the presumptive forebrain whilst Wnt-1, but not Sfrp-2 transcripts, are found at high levels in the developing midbrain (Figs. 3I and 3K). In contrast, Wnt-1 expression overlaps with that of Sfrp-2, but not Frzb, in the anterior neural tube (Figs. 3I, 3J, and 3K; also see Hollyday et al., 1995). By stage 12, Sfrp-2 expression is restricted to the dorsal prosencephalon, hindbrain (rhombomeres 2, 4, and 6), and posterior neural tube (Figs. 3M, 3O, and 3P and data not shown). In contrast to Sfrp-2, transcripts of Frzb are undetectable in the anterior neural tube but, as with Sfrp-2, transcripts are found posteriorly (Figs. 3N, 3Q, and 3S). As earlier, Frzb transcripts are clearly detectable in the cranial neural crest (Fig. 3N) and in addition, Frzb is expressed in the dorsal and ventral streams of trunk neural crest (data not shown). Frzb is also expressed in the lateral plate mesoderm (Figs. 3Q and 3S). By this stage expression of Wnt-8c is downregulated in rhombomere 4 but continues to overlap with Sfrp-2 and Frzb in the posterior neural tube (data not shown). Wnt-1 expression is complementary to both Frzb and Sfrp-2 expression in the neural tube (Figs. 3P, 3Q, and 3R). In the developing brain, Wnt-1 and Sfrp-2 are expressed in overlapping domains (Figs. 3P and 3R).

Expression in the Placodes

Sfrp-2 is expressed in the otic, olfactory, and epibranchial placodes (Figs. 3M, 3P, 4A, and 4D). Expression is found earliest in the otic placode, at stage 11, whilst the olfactory
FIG. 3. Expression of Sfrp-2 and Frzb in the early developing embryo. Whole-mount in situ hybridisation showing the expression of Sfrp-2 (B, I, P), Frzb (C, J, Q), Wnt-8c (D), and Wnt-1 (K, R) in stage 7 (B, C, D), 9 (I, J, K), and 12 (P, Q, R) embryos. (A and E) Vibratome sections through B and C, respectively, showing expression in the neural plate. (F, H and G, L) Sections through the anterior and posterior regions of embryos I and J, respectively, showing dorsally restricted expression in the anterior neural tube (F, G) and Sfrp-2 expression throughout the posterior closing neural tube (H), whereas Frzb expression in this region is negligible (L). (M and N) Sections through rhombomere 6 of the embryos in P and Q, respectively, showing expression of Sfrp-2 in the dorsal neural tube and otic placode and expression of Frzb in the cranial neural crest. (O and S) Sections through the posterior region of embryos P and Q, respectively, showing expression of Sfrp-2 in the neural tube and dermamyotome (O) and Frzb in the lateral plate mesoderm (S; the section is oblique and thus there is expression on the left-hand side only). In B, C, D, I, J, K, P, Q, and R, anterior is uppermost. In the sections the dorsal side is uppermost. d, dermamyotome; h, developing hindbrain; lpm, lateral plate mesoderm; me, mesencephalon; nc, neural crest; np, neural plate; nt, neural tube; op, otic placode; se, surface ectoderm.
and epibranchial placodes express Sfrp-2 by stage 15. Frzb is found in the epibranchial placodes from stage 18 (Fig. 4E).

Expression of Sfrp-2 and Frzb in the Facial Primordia

Initially, Frzb is expressed in the neural crest emanating from the midbrain, as shown by comparison with slug expression (Figs. 4B and 4C), and from the hindbrain (Figs. 3G, 3J, 3N, 3Q, and 4B), whilst Sfrp-2 is expressed in some of the placodes (Figs. 3M, 3P, 4A, and 4D).

By whole-mount in situ hybridisation Sfrp-2 expression was detected throughout the ectoderm of the facial primordia at stage 20 with negligible levels in the mesenchyme (Fig. 4D). By stage 24, there are high levels of mesenchymal expression in a small region of the posterior maxillary primordia and the lateral region of the mandibular primordia (Fig. 4F). In addition, Sfrp-2 is expressed in the mesenchyme in the lateral nasal process (Fig. 4F). By stage 28, Sfrp-2 appears to be associated with a subset of muscles. For example, comparison with MyoD expression shows that Sfrp-2 transcripts are present in the intermandibularis muscle in the mandibular primordia (Fig. 4H and data not shown). In addition, Sfrp-2 transcripts are associated with a muscle medial to the eye (Fig. 4H).

In contrast, Frzb expression is initially more widespread and appears to mark all neural crest-derived mesenchyme. A lateral view at stage 20 (Fig. 4E) shows widespread expression in the facial primordia with an apparent dorsoventral boundary which may correspond to the neural crest–mesoderm boundary (see review by Noden, 1988). Analysis on tissue sections shows that there is a graded expression with Frzb transcripts being more highly expressed in the more distal regions of the developing facial primordia (Fig. 4E and data not shown). As the face develops, Frzb expression becomes more restricted. At stage 24, mesenchymal expression is downregulated in the frontonasal mass compared to the other primordia (Fig. 4G). By stage 28, Frzb is expressed at highest levels in the mesenchyme at the distal tips of the frontonasal mass, throughout the lateral nasal process, and in the medial region of the maxillary primordia (Fig. 4I). In the mandibular primordia, Frzb is expressed in the developing Meckel’s cartilage (Fig. 4I).

Trunk

At stage 9, Sfrp-2 expression is undetectable in the somites but by stage 12, Sfrp-2 transcripts are found throughout the dermamyotome in the more developed somites (Fig. 3O). By stage 20, expression in the dermamyotome is restricted to the small region in the dorsal medial lip in all somites (Figs. 5A and 5C). In addition, with the exception of the somites anterior to the forelimb, which give rise to the tongue muscles, expression is also detectable in the ventral lateral lip of the dermamyotome that gives rise to the muscles of the limb and body wall (Figs. 5A
FIG. 5. Expression of Sfrp-2 and Frzb in the developing trunk. (A and B) Whole-mount in situ hybridisation showing expression of Sfrp-2 in a stage 27 trunk and Frzb in a stage 23 trunk, respectively. (C and D) Vibratome sections through (A) and (B) respectively showing expression of Sfrp-2 in the dorsal medial and ventral lateral lip of the dermamyotome, the myotome, and the migrating muscle precursors (arrowheads), whilst Frzb is in the sympathetic ganglia and dorsal root ganglia together with the ventral roots (long arrow in D). dml, dorsal medial lip; drg, dorsal root ganglia; my, myotome; syg, sympathetic ganglia; vll, ventral lateral lip.
FIG. 7. Expression of Sfrp-2 and Frzb in the developing heart. Whole-mount in situ hybridisation showing expression of Sfrp-2 (A) and Frzb (C) in a stage 24 heart. Section analysis through the ventricle shows restricted expression of Sfrp-2 to the trabeculae (arrowed) and overlying endocardial cell layer (B), whilst a section through the outflow tract and atrioventricular cushions shows Frzb expression in the outflow and atrioventricular cushion mesenchyme (outlined by dashes) and overlying endocardial cells but not in underlying myocardium (D). av, atrioventricular canal; la, left atrium; oft, outflow tract; ra, right atrium; v, ventricle.

FIG. 8. Overexpression of Sfrp-2 and Frzb dorsalises the Xenopus embryo. One ventral blastomere of the four-cell staged Xenopus embryo was injected with 1 ng of RNA. (A) Uninjected stage 36 embryo. (B) Sfrp-2-injected embryo lacking posterior structures (e.g., tail). This embryo also has head defects, which were seen in 25% of the embryos, including poor differentiation of the eye. (C) Frzb-injected embryo lacking posterior structures (e.g., tail). In contrast to Sfrp-2-injected embryos, these embryos did not show head defects. (D) Section of a normal eye from an uninjected embryo. Note the three-layered structure of the retina. (E and F) Sections of abnormal eyes from Sfrp-2- (E) and Frzb- (F) injected embryos. Note the disrupted organisation of the retina. (G–I) Sections through control (G), Sfrp-2-injected (H), and Frzb-injected (I) embryos. The notochord is arrowed in (G–I). Note the highly enlarged notochord in the Sfrp-2-injected embryo.

FIG. 6. Expression of Sfrp-2 and Frzb in the developing limb. Whole-mount in situ hybridisation showing expression of Sfrp-2 (A–D) and Frzb (E–H) in leg buds at stage 20/21 (A, E), stage 23 (B, F), stage 27 (C, G), and stage 30 (D, H). (I–L) Radioactive in situ hybridisation to tissue sections. (I) Transverse section through a stage 28 leg showing expression of Sfrp-2 in the mesenchyme around the muscles. (J and K) Adjacent transverse sections through a stage 24 wing showing expression of Frzb (J) and Sox9 (K) in the central core mesenchyme of the limb bud. (L) Longitudinal section through a stage 32 leg showing Frzb in the perichondrium and epiphyses of the differentiating digits. (I) Photographed under light field; (J–L) photographed under dark field. c, cartilage element; j, joint; m, muscle; pc, perichondrium.
and 5C). Sfrp-2 transcripts are also expressed in the migrating muscle precursors (Fig. 5C).

In contrast, Frzb expression is found in the ventral migrating neural crest in the trunk at stages 9 and 12 with some expression also being detectable in the dorsal streams at stage 12 (data not shown). By stage 20, Frzb is expressed in the sympathetic ganglia and all the dorsal root ganglia, which are derivatives of the ventral migrating neural crest in the trunk, and this expression continues until at least stage 28 (Figs. 5B and 5D).

**Limb Development**

Sfrp-2 is expressed in the proximal mesenchyme in association with developing muscles in the limb bud between stages 20 and 30 (Figs. 6A–6D and 6I). Initially, Sfrp-2 appears to be expressed in the migrating myoblasts, which is consistent with its expression in the ventral lateral lip of the dermamyotome (Figs. 5A and 5C). However, between stages 27 and 30, Sfrp-2 is expressed in the mesenchyme, which contains undifferentiated myogenic cells, and around the developing muscles but not in the differentiated muscles themselves (Fig. 6I). It is difficult to distinguish myogenic precursors from the surrounding mesenchyme. However, the proximal expression of Sfrp-2 correlates with the known location of myogenic precursors, suggesting that the Sfrp-2 expression in the limb probably continues to be restricted to undifferentiated myoblasts (Williams and Ordahl, 1994).

In contrast, Frzb expression is associated with chondrogenesis. Initially, at stage 12, Frzb is expressed weakly in the lateral plate mesoderm and by stage 18, low levels of Frzb are detectable in the developing limb bud (Figs. 3Q and 3S and data not shown). By stage 20, Frzb is highly expressed in the ventral mesenchyme of both fore- and hind-limb buds but by stage 24, expression is localised to the central core of the developing limb bud, which contains the chondrogenic precursors as determined by comparison with Sox9 expression on adjacent sections (Figs. 6E, 6F, 6J, and 6K and data not shown). Similarly at stages 27 and 30, Frzb expression encompasses both prechondrogenic and the surrounding nonchondrogenic mesenchyme (Figs. 6G and 6H). Following differentiation of the chondrocytes into the three chondrogenic layers, Frzb expression becomes restricted to the perichondrium and to the epiphyses of the early developing skeletal elements (Fig. 6L).

**Heart**

Sfrp-2 expression is restricted to the myocardial and endocardial cell layers of the trabeculae in the ventricular compartment between stages 20 and 28 (Figs. 7A and 7B).

Frzb is initially expressed in the endocardial cells of the outflow tract from stage 15 (data not shown). By stage 18, Frzb is expressed in the endocardial and mesenchymal cells throughout the developing atrioventricular canal and the outflow tract cushions and this expression persists until at least stage 26 (Figs. 7C and 7D). The myocardial layers do not express Frzb (Fig. 7D).

**Sfrp-2 and Frzb are Wnt Antagonists**

Sfrp-2 contains a frizzled domain that may directly bind and inhibit members of the Wnt gene family. Since the phenotypes resulting from overexpression of Wnt genes in early Xenopus embryos have been well characterised, we have used this system to test the role of Sfrp-2 in regulating Wnt signalling. Injection of mRNA for members of the Wnt-1 subgroup, including Xwnt-8, dorsalises ventral mesoderm and induces a complete secondary axis when expressed in ventral blastomeres (McAloon and Moon, 1989; Christian et al., 1991; Smith and Harland, 1991; Sokol et al., 1991; Chakrabarti et al., 1992). In contrast, injection of a plasmid containing the Xwnt-8 cDNA under the control of a cytoskeletal actin promoter, such that expression occurs in late blastulae/early gastrulae, ventralises dorsal mesoderm and the resulting embryos lack anterior structures (Christian and Moon, 1993). Injection of mRNA for Frzb, or a dominant-negative Xwnt-8, inhibits the late ventralising activity of Xwnt-8, resulting in hyperdorsalised embryos (Hoppler et al., 1996; Leyns et al., 1997; Wang et al., 1997).

One nanogram of Sfrp-2 or Frzb mRNA was injected into a single ventral blastomere at the four-cell stage and embryos were allowed to develop until stage 36. Localised overexpression of Sfrp-2 on the ventral side of the embryo did not result in the formation of secondary axes, although a few secondary axes (4/30) were seen for Frzb as previously reported (Leyns et al., 1997; Wang et al., 1997). Most injected embryos were hyperdorsalised with a loss of ventro-posterior structures (Figs. 8A, 8B, and 8C), although many Sfrp-2-injected embryos (approximately 25%) also had head defects (Fig. 8B). Injected embryos were scored using the dorsoanterior index (DAI) of Kao and Elison (1988) in which normal embryos have a DAI score of 5 while fully dorsalisated embryos have a DAI score of 10. Sfrp-2-injected embryos had a higher DAI score (DAI = 6.6; N = 35) than Frzb-injected embryos (DAI = 5.8; N = 26), indicating a greater degree of dorsalisation. This was confirmed by sectioning injected embryos, Sfrp-2-injected embryos having a greatly enlarged notochord (Fig. 8H) compared to Frzb-injected embryos (Fig. 8I) and uninjected controls (Fig. 8G). We also noted that the organisation of the retina was disrupted in both Sfrp-2- and Frzb-injected embryos (Figs. 8D, 8E, and 8F). These results are consistent with Sfrp-2 and Frzb inhibiting the ventralising activity of Xwnt-8 during gastrula stages.

We next tested the ability of both Frzb and Sfrp-2 to block the induction of Siamois by Xwnt-8. The transcription factor Siamois has been shown to respond to Wnt signalling in the early Xenopus embryo (Carnac et al., 1996; Brannon et al., 1997). Animal caps from embryos previously injected with either 20 pg of Xwnt-8 mRNA or with 20 pg of Xwnt-8 and 2 ng of either Sfrp-2 or Frzb mRNAs were assayed for
the presence of siamois transcripts using RT-PCR at early gastrula stages (stage 10.5). In control animal caps, very little siamois is detected (Fig. 9, lane 3), while injection of Xwnt-8 RNA induced the expression of siamois (Fig. 9, lane 4) as had been shown by other workers (Carnac et al., 1996). However, coexpression of Xwnt-8 with either Sfrp-2 or Frzb mRNA blocked this induction (Fig. 9, lanes 5 and 6, respectively). These results indicate that both Sfrp-2 and Frzb are able to block Xwnt-8 signalling.

To further test whether Sfrp-2 can inhibit Wnt-mediated dorsalisation we have used a VMZ assay in which Xwnt-8 induces the formation of dorsal mesoderm, including muscle. Both ventral blastomeres were injected at the four-cell stage with either 20 pg of Xwnt-8 mRNA or 20 pg of Xwnt-8 and 2 ng of either Sfrp-2 or Frzb mRNA. VMZs were isolated from early gastrulae (stage 10.5) and allowed to develop until sibling embryos had reached stage 30. Muscle and blood were detected by a double whole-mount immunostaining protocol using the 12/101 anti-muscle monoclonal antibody together with an anti-globin polyclonal antibody (Fig. 10A). VMZs isolated from either uninjected controls (Fig. 10B) or Sfrp-2- or Frzb-injected embryos (data not shown) did not elongate during gastrula stages and show characteristic staining for globin. However, they failed to differentiate any muscle, demonstrating that Sfrp-2 and Frzb have no intrinsic dorsalising activity of their own. In contrast, Xwnt-8-injected VMZs elongated substantially during gastrula stages, indicative of dorsalisation, and muscle was subsequently detected in all such explants, as expected from the known dorsalising activity of this molecule when overexpressed by mRNA injection (Fig. 10C). Globin was never detected. Consistent with a role for Sfrp-2 and Frzb in regulating Wnt function, VMZs co-injected with Xwnt-8 and either Sfrp-2 or Frzb mRNAs exhibited greatly reduced elongation compared to VMZs injected with Xwnt-8 alone (Figs. 10C, 10D, and 10E). Moreover, most co-injected VMZs failed to differentiate any muscle (Figs. 10D and 10E) but the ventral marker globin was expressed in these explants. Our results suggest that Sfrp-2 and Frzb can block the early dorsalising effects of Wnt-8 signalling.

**FIG. 9.** Sfrp-2 and Frzb inhibit induction of Siamois by Xwnt-8. RT-PCR analysis showing siamois and Ef-1α expression in animal caps from embryos injected with Xwnt-8 mRNA alone or together with Sfrp-2 or Frzb mRNAs. Lane 1 is a negative control (a PCR using RNA from a stage 10.5 embryo), whilst lane 2 shows the positive control from cDNA made from the same batch of RNA. Lanes 3–8 show expression in animal caps from control (3) and Xwnt-8- injected (4), Xwnt-8- and Sfrp-2- (5), and Xwnt-8- and Frzb- (6) injected embryos. PCR products were analysed on an agarose gel.

**FIG. 10.** Sfrp-2 and Frzb can inhibit Xwnt-8-mediated dorsalisation. Ventral marginal zone explants were taken from embryos that were injected with the indicated mRNAs, and muscle was detected by whole-mount immunolocalisation using the muscle-specific 12/101 antibody and is stained blue, whilst globin, a ventral marker, is shown by red staining. (A) Control whole embryo showing normal 12/101 localisation in the somites and globin ventrally. (B) Uninjected ventral marginal zone explants do not contain any muscle. (C) Xwnt-8-injected (20 pg) ventral marginal zones differentiate muscle. (D and E) Co-injection of either Sfrp-2 (2 ng) (D) or Frzb (2 ng) (E) blocks most muscle differentiation in Xwnt-8-injected (20 pg) ventral marginal zones.

**DISCUSSION**

Here we describe the cloning and expression of the chick homologues of Sfrp-2 and Frzb. We show that Sfrp-2, as previously reported for Frzb in Xenopus, can function as a Wnt antagonist. The major sites of Sfrp-2 expression are in the developing neuronal structures and regions of myogenesis, whilst Frzb transcripts are localised to four main regions of the embryo—the neural plate, neural crest and some derivatives, areas of chondrogenesis, and the heart. The Wnt genes have a number of different roles during development varying from controlling patterning to proliferation, cell adhesion, and differentiation (reviewed by Parr and McMahon, 1994; Wodarz and Nusse, 1998). The expression patterns of Sfrp-2 and Frzb suggest that these proteins may be important in modulating several of these processes.
Sfrp-2 Is a Wnt Antagonist

Previous studies have shown that Frzb antagonises Wnt signalling and that this is achieved by direct binding between its CRD and Wnts (Bhanot et al., 1996; Lin et al., 1997; Wang et al., 1997; Lescher et al., 1998; Bafico et al., 1999). In this study, functional analysis in Xenopus showed that Sfrp-2, like Frzb, antagonises signalling by Wnt-8. First, injection of mRNA for either Sfrp-2 or Frzb hyperdorsalises Xenopus embryos, but does not dorsalisate ventral marginal zones isolated from early gastrulae or induce secondary dorsal axes when injected ventrally. These results are similar to those previously obtained with RNA transcripts encoding Xenopus Frzb and a dominant-negative mutation of Wnt-8 (Hoppler et al., 1996; Leyns et al., 1997; Wang et al., 1997), both of which inhibit the ventralisating activity of Wnt-8 during gastrulation. Second, injection of mRNA for either Sfrp-2 or Frzb can block the dorsalisating activity of co-injected Wnt-8 mRNA in a ventral marginal zone assay. Thus, the majority of VMZ explants do not express muscle markers. Although we have not directly shown this, our results are consistent with inhibition being mediated by binding of Wnt-8 by Sfrp-2, probably through the CRD.

Overexpression of Frzb and Sfrp-2 disrupted the retina such that it was involuted and disorganised. In the mouse, at least two Wnt molecules, Wnt-7b and Wnt-13, and at least three Wnt antagonists, Sfrp-1, -2, and -4, are expressed in the presumptive retina (Parr et al., 1993; Leimeister et al., 1998; Jasoni et al., 1999). Wnt-13 has been proposed to control/initiate differentiation, whilst Sfrp-2, which is expressed in the proliferating neural cells, has been proposed to control proliferation (Leimeister et al., 1998; Jasoni et al., 1999). This suggests that an interplay of Wnt/Wnt antagonist expression may control proliferation versus differentiation in the developing retina and may be consistent with the eye phenotype that resulted from overexpression of Sfrp-2 or Frzb in Xenopus.

Expression of Sfrp-2 and Frzb

Neuronal development. In a number of regions, Sfrp-2 and Frzb are expressed in developing neuronal structures and differentiated neurones. For example, both are expressed in the early neural plate and developing CNS. In addition Sfrp-2 is found in the otic, epibranchial, and olfactory placodes, which give rise to neurones, whilst in the trunk, Frzb transcripts are found in the dorsal root and sympathetic ganglia derived from neural crest. The expression of Sfrp-2 is similar to that reported in the developing mouse (Leimeister et al., 1998; Lescher et al., 1998).

The Wnts are known to play essential roles during CNS development, controlling both patterning and development of the neuronal structures at a cellular level. Knockout of Wnt-1 function in mice results in the loss of the midbrain and rostral hindbrain (McMahon and Bradley, 1990) with increased apoptosis contributing to some of this loss (Serbedzija et al., 1996), showing that at least one of the Wnts controls the development of specific regions of the CNS. Expression of Sfrp-2 and Frzb overlaps with that of Wnt-1, suggesting that Frzb and Sfrp-2 may modulate Wnt-1 function in specific regions. In addition, these antagonists overlap with Wnt-8c in the most recently formed neural plate/tube, possibly suggesting that downregulation of Wnt-8c function is essential for the progression of neurogenesis. Although to date Wnts have not been shown to have a role in dorsoventral patterning of the neural tube (Ikeya et al., 1997), the differential expression of Wnts across the dorsoventral axis suggests that Wnts may be involved in patterning along this axis. For example, the expression of Wnt-1, Wnt-3, Wnt-3a, and Wnt-4 is restricted dorsally (Roelink and Nusse, 1991; Parr et al., 1993; Holdaway et al., 1995; Ikeya et al., 1997). Sfrp-2 and Frzb expression is regionalised along the anteroposterior and dorsoventral axes of the neural tube, suggesting that they may modify Wnt signalling and hence patterning along these axes. In addition, they may modulate cell proliferation. Knockout of both Wnt-1 and Wnt-3a function in mice results in a decrease in neural crest derivatives and it is possible that Sfrp-2 and Frzb expression modulates levels of Wnt signalling, controlling proliferation of the dorsal neural tube and hence development of the neural crest precursors (Ikeya et al., 1997). Finally, as Wnt signalling from the dorsal neural tube is needed for myogenesis in the adjacent somite, the coexpression of Wnts together with their putative antagonists may block autocrine signalling in the neural tube but allow paracrine signalling to the somites.

In addition, Wnt signalling has been implicated in sensory neurogenesis. At least one member of the Wnt family, Wnt-4, is able to induce the expression of neurotrophin-3, which is necessary for the survival of most sensory neurones, in the limb bud mesenchyme (Patapoutian et al., 1999). Sfrp-2 and neurotrophin-3 are expressed in similar regions in the developing limb bud and Sfrp-2 has been shown to bind Wnt-4 (Lescher et al., 1998; Patapoutian et al., 1999). Thus, in the developing limb bud, Sfrp-2 may modulate Wnt-4 signalling and hence neurogenesis.

Neural crest development. Frzb is expressed in the cranial neural crest that gives rise to the skeletal structures and some neuronal derivatives of the facial primordia and part of the outflow tract of the heart. In the trunk, Frzb is expressed in some neuronal derivatives of the neural crest, i.e., the sympathetic and dorsal root ganglia. In zebrafish, Wnt signalling has been shown to control neural crest differentiation (Dorsky et al., 1998). Expression of Wnt-1 or Wnt-3a promotes pigment differentiation, whereas loss of function of Wnt signalling favours neuronal differentiation (Dorsky et al., 1998). Thus, the expression of Frzb, which can antagonise Wnt-1 signalling in Xenopus, in the neural crest may be important in controlling differentiation. In addition, Frzb activity may modulate proliferation of neural crest/neural crest precursors as discussed earlier.

Sites of epithelial-mesenchymal interactions. In at least two regions of the embryo, the face and limb, Sfrp-2 and Frzb may be involved in epithelial-mesenchymal inter-
actions. Frzb is initially expressed in the ventral mesenchyme of the limb buds and throughout most of the facial mesenchyme. The expression pattern in the early developing limb suggests that Frzb may be involved in dorsoventral patterning, possibly by antagonising the Wnt-7a signal from the dorsal epithelium. However, as Wnt-7a signalling is likely to be restricted to a few cell layers, the significance of the complementary expression patterns of these genes is unclear. It is more probable that Frzb modulates signalling from other Wnts such as Wnt-4 and Wnt-7b, which are expressed in the overlying ventral epithelium (Parr et al., 1993; Patapoutian et al., 1999). The expression of Wnts in the face has not been fully evaluated (reviewed by Francis-West et al., 1998). However, in the mouse, Wnt-3 and Wnt-3a are expressed in the mandibular epithelium overlying Frzb expression (Roelink and Nusse, 1991), whilst Wnt-5a, which is essential for facial outgrowth, is coexpressed in the migrating cranial neural crest and facial mesenchyme (Gavin et al., 1990; Yamaguchi et al., 1999). Frzb cannot antagonise Wnt-3a and Wnt-5a signalling in Xenopus so it is unclear how, and if, these proteins would interact. However, there is some evidence that Frzb can enhance the effects of Wnt-5a at least in Xenopus (Lin et al., 1997). Sfrp-2 may also modulate epithelial–mesenchymal interactions. In particular, Sfrp-2 in the limb bud may modulate Wnt induction of sensory neurogenesis as discussed above and possibly myogenesis as discussed below.

**During chondrogenesis.** Frzb is expressed in precondensing mesenchyme in the limbs and face as previously reported in humans and mice (Hoang et al., 1996, 1998). A number of Wnts have been shown to block chondrogenic differentiation in vivo and in vitro (Rudnicki and Brown, 1997; Kawakami et al., 1999). Thus, Frzb may promote cartilage development by blocking Wnt activity. However, to date, the complete expression of Wnts during cartilage development has not been reported, although Wnt-11 has been shown to be expressed in the developing perichondrium in humans (Lako et al., 1998). Therefore, how Wnt/Frzb interactions control chondrogenesis has yet to be elucidated.

**During myogenesis.** In several regions of the developing embryo, the expression of Sfrp-2 appears to be associated with myogenesis. In the somites, Sfrp-2 expression is found in the dorsal medial and ventral lateral lips of the dermamyotome, whilst in the limb bud and developing face, Sfrp-2 is expressed in the myogenic regions.

Muscle development has been most well characterised in the somites where Wnt signalling from the dorsal ectoderm and dorsal neural tube, together with Sonic hedgehog signalling, is essential for the development of the myotome (Münsterberg et al., 1995; Stern et al., 1995; Fan et al., 1997; Hirsinger et al., 1997; Marcelle et al., 1997; Capdevila et al., 1998; Ikeya and Takada, 1998). To date, Wnt-1, Wnt-3, Wnt-4, Wnt-5a, Wnt-6, and Wnt-7a have all been shown to be able to induce myogenesis, to varying degrees, in explanted somites, consistent with the expression of Wnts in the dorsal ectoderm and the dorsal neural tube (Münsterberg et al., 1995; Stern et al., 1995; Tajbakhsh et al., 1998). Furthermore, it has been proposed that different Wnts may activate distinct myogenic pathways (Tajbakhsh et al., 1998). The expression of a putative antagonist, Sfrp-2, in the developing myotome, in association with Wnt-6 and Wnt-11 in the medial lip, would appear to be contradictory to the muscle-inducing ability of Wnts (Parr et al., 1993; Christiansen et al., 1995; Tanda et al., 1995; Marcelle et al., 1997). However, Sfrp-2 may prevent precocious differentiation of the developing myoblasts until they have reached their destination or may selectively antagonise one group of Wnts promoting one myogenic pathway. Support for the former idea has recently been obtained for Frzb, which is expressed in the myotome in mice. Overexpression of Frzb in developing somites blocks expression of Myf-5 and MyoD, determinants of myogenic differentiation (Borello et al., 1999). Later in development, in the face and limb, Sfrp-2 is expressed in regions of myogenesis in association with Wnt-11 expression, where again, Sfrp-2 may modulate muscle differentiation (Christiansen et al., 1995; Tanda et al., 1995; Kawakami et al., 1999).

Wnts control development by a variety of mechanisms such as by modulating cell adhesion, proliferation, cell transformation, and differentiation. The Wnt antagonists are expressed in a number of regions where they may act to modulate these processes. For example, the expression of Frzb in the cardiac cushions suggests that Wnt signalling may be involved in epithelial–mesenchymal transformation, whilst its expression in condensing mesenchyme may increase cell adhesion essential for the initial steps of chondrogenesis. The most striking expression pattern of Sfrp-2 is in the neuronal structures, such as the neural plate and some of the placodes, and in regions of muscle development in the somites and limb. In these regions Sfrp-2 expression may control proliferation and/or differentiation. Further investigations will fully elucidate the role of Sfrp-2 and Frzb in these processes. The role of the Sfrps may not be just the direct antagonism of Wnt proteins. Recent work has suggested that these Wnt antagonists may also act as agonists (Lin et al., 1997), increasing the complexity of these signalling interactions.

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