INTRODUCTION

Gastrulation is a highly complex and tightly regulated process that involves polarised changes in cell shape, directed cell migration, tissue rearrangements and modulation of the cell cycle: all of which occur while cells receive inductive signals informing them of their fate in the embryo. Recently, thanks to studies in Xenopus and zebrafish embryos, our understanding of vertebrate gastrulation has improved significantly (Heisenberg et al., 2000; Myers et al., 2002; Tada and Smith, 2000; Wallingford et al., 2002). For example, we now know that Wnt signalling, acting through the non-canonical planar polarity pathway, regulates convergent extension during gastrulation in Xenopus and zebrafish embryos (Wallingford et al., 2002). Thus, Dishevelled constructs, which specifically disrupt planar cell polarity signalling, interfere with convergent extension in both Xenopus (Tada and Smith, 2000; Wallingford et al., 2002) and zebrafish (Heisenberg et al., 2000), as does disruption of the functions of frog and fish homologues of Strabismus (Van Gogh – FlyBase), a Drosophila gene involved in planar cell polarity (Darken et al., 2002; Goto and Keller, 2002; Park and Moon, 2002; Wolff and Rubin, 1998). Other components of the Wnt planar cell polarity pathway, such as RhoA and JNK, regulate cytoskeletal function, and thereby cell shape and polarity (Habas et al., 2001).

In addition, the roles of cell adhesion molecules, such as laminin (Nakatsuji, 1986) and fibronectin (Marsden and DeSimone, 2001; Reintsch and Hausen, 2001; Winklbauer and Keller, 1996), in the regulation of gastrulation are becoming clearer, as are the functions of their receptors, including the integrins (Davidson et al., 2002; Ramos et al., 1996; Whittaker and DeSimone, 1993). The successful prosecution of gastrulation and cell fate specification requires the coordination and integration of all these activities. In an attempt to shed light on this issue, we have focused on the cysteine-rich secreted protein Cyr61, a member of the CCN (Cyr61, CTGF, Nov) family. Members of the CCN family are versatile proteins, exhibiting properties that might well be expected of key regulators of gastrulation: they associate with the extracellular matrix; they can mediate cell adhesion, cell migration and chemotaxis; and they can augment the activity of peptide growth factors (Lau and Lam, 1999). Significantly, Cyr61 can also induce signalling events, such as activation of ERK and Rac, and the induction of gene expression in fibroblasts (Chen et al., 2001a; Chen et al., 2001b).

Members of the CCN family have four characteristic domains, each encoded by a separate exon, and each of which is defined by similarities to other families of secreted proteins (Fig. 1A) (Bork, 1993). The first domain, which follows the secretory sequence, is similar to the IGF-binding domain of insulin-like growth factor binding proteins (IGFBPs). The second region, usually referred to as the von Willebrand factor C (VWC) domain, resembles the cysteine-rich domains of chordin, a BMP antagonist (Sasai et al., 1994). The third
domain has homology to the thrombospondin (TSP) type I repeat. The fourth, or C-terminal (CT), region contains cystine knot domains (Vitt et al., 2001) and is characterised by its similarity to slit proteins (Bork, 1993), which are involved in axonal pathfinding (Brose and Tessier-Lavigne, 2000; Rothberg et al., 1988). A region of variable sequence and length is positioned between the VWC and TSP domains.

We show that *Xenopus* Cyr61 is expressed maternally and that zygotic activation occurs at late neurula stages. Interference with the early function of *Xenopus* Cyr61 by antisense morpholino oligonucleotides disrupts gastrulation, as does overexpression of Xcyr61 RNA and intrablastocoellic injection of purified Cyr61 protein. Our results suggest that Xcyr61 regulates gastrulation through its ability to modulate assembly of the extracellular matrix of the blastocoel roof, as well as by mediating cell adhesion in a heparan-sulphate proteoglycan-dependent manner. It is also likely that Xcyr61 modulates gastrulation through its ability to modulate the Wnt pathway: Xcyr61 can, under different circumstances, either antagonise or stimulate Wnt signalling. The CT domain, which shares homology with slit proteins, is necessary and sufficient for antagonism of Wnt signalling but not for its stimulation. It is possible that Xcyr61 modulates other signalling pathways, and we present evidence that, like the CCN family member CTGF (Abreu et al., 2002), it might also act as a weak antagonist of BMP signalling. Together, these findings suggest that Xcyr61 is an important regulator of gastrulation movements and of multiple signalling pathways during early development.

**MATERIALS AND METHODS**

*Xenopus* embryos and microinjection

*Xenopus laevis* embryos were obtained by artificial fertilisation as described previously (Smith and Slack, 1983). They were staged according to Nieuwkoop and Faber (Nieuwkoop and Faber, 1975). Microinjection of RNA was carried out as previously described (Smith, 1993). Embryos were cultured in 10% normal amphibian medium (NAM) (Slack, 1984) and tissue explants were cultured in 75% NAM. Mouse Cyr61 protein, or bovine serum albumin as a control, was injected at a concentration of 0.2 mg/ml in the buffer described by Kireeva and colleagues (Kireeva et al., 1996).

cDNA isolation and plasmid construction

Sequence encoding *Xenopus* Cyr61 (Xcyr61) was isolated by the polymerase chain reaction (PCR) using degenerate primers based on the conserved cysteines, in domains 2 and 3 of mammalian Cyr61 proteins, that flank the central variable region. Primers used were:

- upstream, 5′-CCGGAATTCGGGAGAAGATGAACCAATG-3′;
- downstream, 5′-CTGAGCTGAGTTCGAGG-3′;
- and 5′-CTGGGATCCTCGACGATGACCTTGGC-3′;

The resulting fragment was used as a probe to isolate a near-full-length cDNA from a tadpole cDNA library (GenBank Accession Number AF320592). This fragment was also used as a probe in RNAase protection assays. Primer extension analysis suggested that this cDNA lacked ~100 nucleotides from the 5′ end (data not shown; full details are available on request).

The Xcyr61 open reading frame was cloned into the vectors pSP64T and pcDNA3. Deletion constructs comprising domains 1, 2 and 3, domains 1 and 2, and domain 1 alone were created by PCR using standard techniques; they were then inserted into pSP64T or pcDNA3. A construct comprising the CT domain alone (domain 4) was created by two rounds of PCR. This construct consisted of sequence preceding domain 1, including the secretory signal, fused in frame to domain 4.

Other expression plasmids were as follows: Xwnt8 (Sokol et al., 1991), Dsh (Sokol et al., 1995), ddl (Sokol, 1996) and β-catenin (Domingos et al., 2001).

Adhesion/cell spreading assay

Lab-Tek chamber slides (Nalge Nunc International) were coated overnight at 4°C, or for 3-4 hours at room temperature, with 50 μg/ml fibronectin (Sigma), 10 μg/ml mouse Cyr61 (Kireeva et al., 1996) or 10 μg/ml human Cyr61ΔCT (Grzeszkiewicz et al., 2001) dissolved in NAM containing 7.5% of the normal divalent cation concentration (LCMM) and 0.1% bovine serum albumin (BSA). Adhesive surfaces were blocked with LCMM containing 0.1% BSA for 1 hour at room temperature. Animal pole explants were dissociated in Ca2+- and Mg2+-free medium (CMFM) containing 0.1% BSA, and the cells were plated at a density of approximately two animal pole equivalents per chamber in LCMM/0.1% BSA containing 10 U/ml activin (Cooke et al., 1987). Heparin (Sigma) was used at a final concentration of 10 μg/ml. Cell spreading was documented by photomicrography. Cells were subsequently fixed in MEMFA and stained with phalloidin/4′,6-diamidino-2-phenylindole (Sigma) for observation by confocal microscopy.

Antisense morpholino oligonucleotides

These were purchased from GeneTools, LLC (Oregon, USA). Sequences were:

- MO1, 5′-AGCAAAACTGGCAAAATACTGAAAG-3′;
- MO2, 5′-AGCAAAACTGGCAAAATACTGAAAG-3′;
- MO3, 5′-TAAACTGGCCAGATGCTTCTCTG-3′;
- Con 1, 5′-CTCTCTACCTGATTTAATTTAAT-3′;
- Con 2, 5′-GAACTGGTTTGTGTTTGTGTT-3′; and
- Con 3, 5′-GTACAGCAGTTGTTTGTGTT-3′.

All morpholino oligonucleotides were dissolved at 3 μg/ml in 5 mM HEPES (pH 7.6).

Whole-mount in situ hybridisation and immunostaining

In situ hybridisation and immunohistochemistry were carried out essentially as described (Harland, 1991), using a hydrolysed Xcyr61 cDNA (see above), cardiac actin (Mohun et al., 1984) or XAG-1 (Sive et al., 1989) cDNAs, or anti-mouse Cyr61 (Kireeva et al., 1997) or anti-muscle 12/101 (Kintner and Brockes, 1984) antibodies.

Scanning electron and confocal microscopy

Scanning electron microscopy of *Xenopus* embryos was carried out as described (Howard et al., 1992). Confocal microscopy was carried out using a Leica scanning laser microscope.

RNAase protection assays

RNA was prepared using the acid guanidinium thiocyanate-phenol-chloroform method (Chomczynski and Sacchi, 1987). RNAase protection analysis was carried out essentially as described by Jones and colleagues (Jones et al., 1995), using RNAase T1 alone for all probes. An Xcyr61 probe was made using the original Xcyr61 PCR fragment, which was cloned into pBluescript KS. This plasmid was linearised with EcoRI and transcribed with T7 RNA polymerase. Probes for siamois (Lemaire et al., 1995) and ornithine decarboxylase (Isacs et al., 1992) were as described.

Luciferase assays

Luciferase assays were performed using the Promega Dual-Luciferase assay kit. Embryos were injected with 10 pg TOPFLASH (van de Watering et al., 1997), 10 pg pRL-SV40/TK as a reference plasmid, and an appropriate amount of RNA encoding components of the canonical Wnt signalling pathway. Animals caps were dissected at stage 8.5 and cultured in 75% NAM for 3-4 hours. They were then suspended in 10 μl of 1× Passive Lysis Buffer per cap and, after
centrifugation, 5 µl was taken for assay. All values were expressed as Relative Luciferase Units (Firefly luciferase activity/Renilla luciferase activity), with the value for the DNA alone sample being set at unity. Each experiment shown was carried out at least three times.

**Western blotting**

Western blots were carried out as described (Tada et al., 1997), using antibodies raised against fibronectin (Marsden and DeSimone, 2001) or against Hsp70 (Sigma).

**RESULTS**

**Xenopus Cyr61**

We isolated a *X. laevis* cDNA encoding a protein with 81% identity to human and 77% identity to mouse Cyr61 (Fig. 1A,B; GenBank Accession Number AF320592), and ~50% identity to other members of the CCN family, such as CTGF (not shown). This suggested that we had cloned *Xenopus Cyr61* (*Xcyr61*). An EST sequence with high homology to the 5' region of *Xcyr61* was found in GenBank (Accession Number BF048680). The two sequences differ in a few 5' nucleotides, and may be a consequence of the tetraploid nature of the *X. laevis* genome. When necessary we refer to the former allele as *Xcyr61a* and the latter as *Xcyr61b*.

**Fig. 1.** Sequence and expression pattern of *Xenopus Cyr61*. (A) Domain structure of Cyr61. IGFBP, insulin growth factor binding protein domain; VWC, von Willebrand type C domain (also referred to as the cysteine rich domain of Chordin and short gastrulation); TSP, thrombospondin domain; CT, carboxy-terminal domain with homology to the neuronal pathfinding protein slit. (B) Alignment of Cyr61 proteins from chick, *Xenopus*, rat and human. Note the high degree of conservation throughout the protein, except in the signal peptide and the variable central region. (C) Temporal expression pattern of *Xcyr61* mRNA assessed by RNAase protection assay. Transcripts are present maternally and persist at least until early blastula stage 6, when they are present in both the animal (lane 6) and vegetal (lane 7) hemispheres of the embryo. Expression is then activated zygotically from mid-neurula stage 14 (lane 9). Ornithine decarboxylase (ODC) is used as a loading control. (D-F) Whole-mount in situ hybridisation analysis of *Xcyr61* expression. At stage 28 (D), expression is detectable in the somites and branchial arches. A cleared embryo (E) reveals expression in the notochord, an observation that was confirmed in sectioned embryos (data not shown). At stage 34 (F), transcripts are present in the posterior cardinal vein (arrow). Sections of embryos such as these show that expression of *Xcyr61* in the somites is concentrated in and around the nuclei, which suggests that transcripts are unstable (not shown). (G-I) Immunofluorescence analysis of the distribution of exogenous mouse Cyr61 in *Xenopus* gastrulae. (G) An uninjected embryo at early gastrula stage 10 does not react with a mouse Cyr61 antiserum. (H) An embryo previously injected with RNA encoding mouse Cyr61 reveals accumulation of mCyr61 in the blastocoel roof at the early gastrula stage (arrows). (I) *Xenopus* fibronectin also accumulates in the blastocoel roof (arrow). Note that expression of *Xcyr61* during gastrulation proper is very low; this suggests that our morpholino oligonucleotides (Fig. 3) are targetting translation of maternal *Xcyr61* mRNA. Scale bars: D, 0.4 mm; E, 0.25 mm; F, 0.4 mm; G, 0.25 mm; H, 80 µm; I, 40 µm.
In an effort to overcome this problem and to examine the localisation of Cyr61 protein, we injected RNA encoding mouse Cyr61 into *Xenopus* embryos at the one-cell stage and detected the resulting protein using a well characterised antibody directed against mouse Cyr61 (Kireeva et al., 1997). Surprisingly, despite the widespread distribution of the injected RNA (data not shown), Cyr61 protein was detectable only in the roof of the blastocoel at late blastula and early gastrula stages, in a pattern resembling that of fibronectin (Fig. 1G-I). This suggests that the localisation of Xcyr61 is regulated in some way.

**Overexpression of Xcyr61 interferes with gastrulation movements**

Overexpression of Xcyr61 by RNA injection into the one-cell stage embryo caused gastrulation defects (Fig. 2A,B). In particular, there was a severe delay of blastopore closure, tissue appeared to accumulate around the blastopore, and embryos did not elongate fully along the anteroposterior axis. As discussed below, these defects may result from disruption of epiboly and of convergent extension movements.

Does ectopic Xcyr61 act at early stages, perhaps in regional specification, or does it exert its effects during gastrulation itself? To address this question, we injected mouse Cyr61 protein directly into the blastocoels of embryos at the early gastrula stage. Such embryos displayed gastrulation defects that are indistinguishable from the phenotype produced by injecting mRNA (Fig. 2D,E,F), which suggests that Cyr61 does not disrupt gastrulation movements by modulating early inductive events but rather through a more direct effect on the extracellular events that occur during gastrulation itself. Injection of bovine serum albumin, in the same buffer, had no effect (Fig. 2D).

These effects were investigated in more detail by studying isolated dorsal marginal zone tissue. Dissected dorsal marginal zone explants undergo gastrulation movements, including epiboly and convergent extension, in a manner that resembles their behaviour in the embryo (Keller and Danilchik, 1988). By contrast, dorsal marginal zone regions derived from embryos injected with Xcyr61 mRNA gastrulated abnormally. In particular, epiboly was severely disrupted because the pigmented ectodermal cells that normally cover the yolky mesendodermal tissue (Fig. 2G) failed to do so (Fig. 2H).

**Antisense oligonucleotides directed against Xcyr61 also cause gastrulation defects**

To assess the role of Xcyr61 in early development in more detail, we designed and tested three antisense morpholino oligonucleotides (Fig. 3A). Antisense morpholino oligonucleotides have been shown to block translation of their target RNAs efficiently in both *Xenopus* and zebrafish embryos (Heasman et al., 2000; Nasevicius and Ekker, 2000). The first morpholino oligonucleotide (MO1) was directed against Xcyr61a and the second (MO2) against Xcyr61b. The two morpholino
sequences differed by only one base and it seemed likely that each would interfere with the translation of both Xcyr61 alleles. Indeed, as we describe below, the two oligonucleotides gave identical phenotypes following injection into Xenopus embryos. However, to confirm the specificity of the two oligonucleotides we also designed MO3, which matches both Xcyr61a and Xcyr61b and does not overlap with MO1 and MO2. All three antisense morpholino oligonucleotides inhibited in vitro translation of Xcyr61a in a specific manner (Fig. 3B). Injection of any of the three Xcyr61 antisense morpholino oligonucleotides caused defects in gastrulation that resembled those caused by overexpression of Cyr61 (Fig. 2B,C). Thus, epiboly was disrupted in dorsal marginal zone explants (Fig. 2I) and, in intact embryos, we observed a severe delay of blastopore closure (Fig. 4A,B; data not shown). There was no gross failure of germ layer patterning in the embryo because Xbra was expressed throughout the marginal zone of injected embryos (Fig. 4C,D) and goosecoid was expressed in the dorsal marginal zone (Fig. 4E,F). However, we note that expression of Xbra was reduced compared with controls (Fig. 4C,D). We do not yet know if this was a consequence or a cause of the defect in gastrulation; this is discussed below. We also observed that the Xbra expression domain was positioned closer to the equatorial region of the embryo than it was in controls, and that the goosecoid domain was broader (Fig. 4C-F). These phenomena are likely to be direct consequences of the disruption of gastrulation, although we cannot rule out subtle effects on regional specification.

Although blastopore closure was impaired in embryos injected with Xcyr61 antisense morpholino oligonucleotides, many went on to develop clear dorsoventral and anteroposterior axes with cement glands at their anterior ends (Fig. 4G,H). All 51 embryos examined contained a notochord (data not shown), but they were shortened and tail formation was abnormal, perhaps again a result of the disruption of gastrulation. Many of these embryos appeared otherwise normal, but some had small eyes or lacked eyes completely (Fig. 4H). Bisection of gastrula-stage embryos injected with Xcyr61 antisense morpholino oligonucleotides revealed changes in the structure of both the marginal zone and the animal pole region.

**Fig. 4.** Antisense morpholino oligonucleotides directed against Xcyr61 inhibit gastrulation movements but have little effect on mesodermal specification. (A,B) Morpholino oligonucleotide MO1 causes a severe retardation in blastopore closure (B) compared with control stage 12 embryos (A). (C,D) Morpholino oligonucleotide MO1 causes a decrease in Xbra expression and shifts the Xbra expression domain towards the animal pole. Embryos are at stage 11.5. (E,F) Morpholino oligonucleotide MO1 causes expansion of the goosecoid expression domain. Embryos are at stage 11.5. (G,H) Morpholino oligonucleotide MO1 (30 ng) causes shortening of the anteroposterior axis. Embryos are at stage 35. (I-K) Bisection of embryos injected with morpholino oligonucleotide MO1 reveals changes in the structure of the blastocoel roof and of the marginal zone. (I) Embryo previously injected with a control morpholino oligonucleotide at stage 11. The blastocoel roof and marginal zone are thin and compact, as indicated by the two white lines. (J,K) Morpholino oligonucleotide MO1 (30 ng) causes a thickening of the blastocoel roof and marginal zone (lines), and a separation of cell layers (arrows). (L,M) Scanning electron microscope images of a control embryo at stage 11 (L) and an embryo at the same stage previously injected with 30 ng antisense morpholino oligonucleotide MO1 (M). Note the tightly packed epithelial appearance of the cells in the blastocoel roof of the control embryo (L), and the more loosely packed appearance of cells in the MO1-injected embryo, with some cells apparently about to detach (arrows; M). Note also that the migration of the large flat mesendodermal cells visible at the bottom of (L) is impaired in MO1-injected embryos (M). (N,O) Morpholino oligonucleotide MO2 causes a decrease in fibronectin assembly in the blastocoel roof. (N) Fibronectin forms an elaborate fibrillar network in the blastocoel roof of control embryos. (O) Fibronectin assembly is reduced in the blastocoel roof of morpholino-injected embryos. (P) Western blot analysis indicates that levels of fibronectin are similar in control and morpholino-injected embryos. HSP-70 was used as a loading control. Scale bars: in L, 100 μm for L,M; in O, 100 μm for O,N.
The superficial and deep layers of the marginal zone, which are usually tightly adherent, became separated and their constituent cells were more loosely packed (Fig. 4I-K). The animal pole regions of injected embryos were thicker than those of controls (Fig. 4I-K). Failure of the animal pole region to undergo thinning during gastrulation suggested that epiboly and radial intercalation was disrupted in such embryos. Cells in the animal pole regions of these embryos appeared rounder, less adherent and, as in the marginal zone, more loosely packed (Fig. 4I-K). Scanning electron microscopy confirmed these impressions (Fig. 4L,M), and also indicated that migration of large flat mesendodermal cells, visible at the bottom of Fig. 4L, was impaired in MO1-injected embryos (Fig. 4M). Similar results have been obtained with morpholino oligonucleotide MO2. These changes in the animal pole blastomeres are likely to be caused by a decrease in cell adhesion rather than by apoptosis: we observed no significant increase in TUNEL-staining cells in embryos injected with either MO2 or MO3 (data not shown).

The effects of the morpholino oligonucleotides on the structure of the animal cap are consistent with the idea that the downregulation of Xbra observed in Fig. 4D was not the primary cause of the gastrulation defect illustrated in Fig. 4, because Xbra is not expressed in the animal hemisphere. Rather, it seems likely that gastrulation was disrupted, at least in part, because Xcyr61 protein did not accumulate in the roof of the blastocoel. This may in turn disturb the distribution of other components of the extracellular matrix. For example, in control embryos, fibronectin forms an elaborate fibrillar network in the blastocoel roof (Fig. 4N) and this acts as a substrate for the adhesion and migration of involuted mesoderm cells (Marsden and DeSimone, 2001). By contrast, in MO2-injected embryos, there was a dramatic reduction in the amount of fibronectin in the extracellular matrix of the blastocoel roof, and the remaining fibrils appeared disorganised (Fig. 4O; data not shown). The apparent reduction in levels of extracellular fibronectin is likely to be caused by a defect in fibronectin fibril assembly rather than by a decrease in overall levels: western blotting experiments indicated that levels of fibronectin were similar in control and morpholino-injected embryos (Fig. 4P).

The similar phenotypes produced by the three antisense morpholino oligonucleotides, two of which are non-overlapping, suggests that their effects are specific. The specificity of MOs 1-3 was examined further by performing ‘rescue’ experiments. Such experiments are difficult to interpret when, as here, overexpression of a gene product produces a phenotype similar to that observed following inhibition of its function; an observation that, in our experiments, suggests that normal gastrulation requires both precisely controlled levels and localisation of Xcyr61. Nevertheless, we observed that whereas 91% of MO2-injected embryos exhibited either the more severe ‘small eyes’ or ‘gastrulation defect’ phenotype, co-injection of 200 pg Xcyr61 RNA reduced this figure to 50% (Fig. 5). These observations also suggest that the effects of the antisense morpholino oligonucleotides are specific.

**Xcyr61 promotes heparan sulphate proteoglycan-mediated adhesion of gastrula-stage cells**

How might depletion, or overexpression, of Xcyr61 disrupt gastrulation? Several biological activities have been attributed to Cyr61, including the promotion of cell adhesion and migration, and the ability to cooperate with growth factors (Kireeva et al., 1996). Both of these activities might be involved in the regulation of gastrulation.

We first used activin-treated animal pole cells (Smith et al., 1990) to show that purified mouse Cyr61, like fibronectin, can support cell adhesion during gastrulation (Fig. 6). Significantly, cells adherent to Cyr61 proved to have a different shape from those adherent to fibronectin (Fig. 6A,B). The latter were usually polarised, with prominent filopodia and fewer lamellipodia (Fig. 6A), whereas cells adherent to Cyr61 were characterised by large lamellipodia frequently distributed in a near-symmetrical fashion around the cell (Fig. 6B). Because cell migration requires the dynamic formation and disappearance of lamellipodia and associated focal adhesions, it is possible that cells adherent to Cyr61 are not as motile as those adherent to fibronectin. Defects in gastrulation might therefore arise from either depletion or overexpression of Xcyr61.

Like other members of the CCN family, Cyr61 consists of four protein domains (Fig. 1A). The CT domain mediates adhesion to fibroblasts (Grzeszkiewicz et al., 2001) and we found that it also mediates adhesion of cells from gastrula stage *Xenopus* embryos: purified Cyr61 protein lacking the CT domain cannot support stable adhesion and spreading of these cells, and they resembled those seeded onto bovine serum albumin (BSA; Fig. 6C-E). Fibroblast adhesion to Cyr61 also requires heparan sulphate proteoglycans (HSPGs) as co-receptors (Chen et al., 2000), and consistent with this observation we find that exogenous heparin, which competes...
oligonucleotides were loosely packed and apparently less adherent (Fig. 4I-M). To determine whether this was associated with a decrease in cell adhesion, cells from the blastocoel roofs of control embryos or of embryos injected with antisense morpholino oligonucleotides were dissociated by culture in Ca\(^{2+}\) and Mg\(^{2+}\)-free medium and then reaggregated by addition of Ca\(^{2+}\) (Torres et al., 1996). Control cells formed large aggregates within minutes (Fig. 6I), whereas those derived from embryos injected with antisense morpholino oligonucleotides formed only small cell groups (Fig. 6J), which suggests that their capacity to form Ca\(^{2+}\)-dependent contacts was compromised.

**Cyr61 can induce secondary axes in the Xenopus embryo**

The results described above suggest that Xcyr61 regulates gastrulation through its influence on cell-cell and cell-matrix adhesion, but it is also possible that it influences cell signalling, perhaps through cooperation with growth factors. We note, for example, that depletion of Xcyr61 caused downregulation of Xbra expression (Fig. 4C,D), and mammalian Cyr61 has been shown to cooperate with several growth factors in vitro and to induce changes in gene expression and cell morphology (Chen et al., 2001a; Chen et al., 2001b).

To examine the ability of Xcyr61 to influence early embryonic signalling, we first injected Xcyr61 mRNA into ventral or dorsal blastomeres of Xenopus embryos at the four-to-eight-cell stage. Dorsal injections resulted in the formation of embryos with enlarged heads, which suggests that Xcyr61 has dorsalisising activity (data not shown), and, consistent with this, ventral injections caused secondary axis formation (Fig. 7A). In a few cases (1-5%, depending on the egg batch) complete secondary axes were induced (Fig. 7B), but more frequent were partial secondary axes (5-30%; Fig. 7C,D) or the induction of ectopic muscle (30-80%; Fig. 7E,F). In addition, most injected embryos had blastopore closure defects, perhaps reflecting the ability of Xcyr61 to regulate gastrulation movements.

In order to determine which domains of Xcyr61 mediate its dorsalisising activity, we tested three deletion constructs: one of which contained the IGFBP domain alone (construct 1); one the IGFBP and VWC domains (construct 1,2); and one the IGFBP, VWC, variable and TSP domains (construct 1,2,3). The IGFBP domain proved to be sufficient to cause dorsalisation (data not shown), and, consistent with this, ventral injections caused secondary axis formation (Fig. 8E). To examine the ability of Xcyr61 to influence early embryonic signalling, we first injected Xcyr61 mRNA into ventral or dorsal blastomeres of Xenopus embryos at the four-to-eight-cell stage. Dorsal injections resulted in the formation of embryos with enlarged heads, which suggests that Xcyr61 has dorsalisising activity (data not shown), and, consistent with this, ventral injections caused secondary axis formation (Fig. 7A). In a few cases (1-5%, depending on the egg batch) complete secondary axes were induced (Fig. 7B), but more frequent were partial secondary axes (5-30%; Fig. 7C,D) or the induction of ectopic muscle (30-80%; Fig. 7E,F). In addition, most injected embryos had blastopore closure defects, perhaps reflecting the ability of Xcyr61 to regulate gastrulation movements.

Induction of complete secondary axes in Xenopus embryos can be achieved by activation of the canonical Wnt signalling pathway. Consistent with the idea that Xcyr61 acts through the Wnt pathway, we observe that Xcyr61 mRNA can induce weak expression of Siamois, a direct target of the Wnt/β-catenin pathway, in ventral marginal zone tissue (Fig. 7G). To confirm that induction of Siamois by Xcyr61 can lead to stable dorsalisation of ventral marginal zone tissue, we examined such explants at tadpole stage 32 for expression of the muscle marker cardiac actin. Both full-length Xcyr61 and a deletion construct comprising just the IGFBP and VWC domains (construct 1,2) induce expression of cardiac actin (Fig. 7H-L), confirming that Xcyr61 can dorsalisate ventral marginal zone tissue.

Xcyr61 also induced the TOPFLASH synthetic reporter, which responds directly to Wnt/β-catenin signalling, in ventral

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**Fig. 6.** Cyr61 promotes CT domain- and heparan sulphate proteoglycan-dependent spreading of cells from blastulae; Xcyr61 is required for cell-cell adhesion. (A,B) Confocal microscope images of phalloidin-FITC stained activin-treated animal pole blastomeres spreading on fibronectin (A) or purified mouse Cyr61 (B). Cells plated on fibronectin have a polarised phenotype, with a least one long filopodium and, at the opposite end of the cell, lamellipodia. Cells plated on Cyr61 are characterised by extensive lamellipodia and no filopodia. (C-H) Phase-contrast images of live activin-treated animal pole blastomeres seeded on bovine serum albumin (BSA), fibronectin (FN), Cyr61 (CYR61) or Cyr61 lacking the CT domain (–CT) in the absence (C,D,E,G) or presence (F,H) of heparin (H). Cell spreading on Cyr61 requires the CT domain of that protein and is inhibited by heparin. Cell spreading on fibronectin is not inhibited by heparin. (IJ) Re-aggregation of blastomeres requires Xcyr61. Blastocoel roofs derived from control embryos or from embryos injected with MO2 (30 ng) were dissociated and allowed to re-aggregate. Cells derived from control embryos formed large clumps (I); those derived from MO2-injected embryos re-aggregated poorly (J). Scale bars: in B, 20 μm for A,B; in C, 100 μm for C-H; in I, 300 μm for I,J. with the cell-associated HSPGs for sites on Cyr61, blocks adhesion of Xenopus cells to Cyr61 (Fig. 6E,F), whereas adhesion to fibronectin is unaffected (Fig. 6G,H). Interestingly, depletion of HSPGs also disrupts gastrulation in intact Xenopus embryos (Brickman and Gerhart, 1994; Itoh and Sokol, 1994), as does mutation of the zebrafish glypcian knypek (Topczewski et al., 2001).

We noted that cells of the blastocoel roof and marginal zone of embryos injected with Xcyr61 antisense morpholino mRNA can lead to stable dorsalisation of ventral marginal zone tissue, we examined such explants at tadpole stage 32 for expression of the muscle marker cardiac actin. Both full-length Xcyr61 and a deletion construct comprising just the IGFBP and VWC domains (construct 1,2) induce expression of cardiac actin (Fig. 7H-L), confirming that Xcyr61 can dorsalisate ventral marginal zone tissue.

Xcyr61 also induced the TOPFLASH synthetic reporter, which responds directly to Wnt/β-catenin signalling, in ventral
marginal zone tissue (Fig. 7L). The level of TOPFLASH induction by Xcyr61 was modest compared with that obtained with Xwnt8 (not shown) or Dishevelled (Fig. 7L), consistent with the observation that Xcyr61 usually induces incomplete secondary axes. A Xcyr61 construct comprising just the IGFBP domain also activated the TOPFLASH reporter, and with higher activity than the full-length protein. Xcyr61 is likely to activate TOPFLASH by acting through the canonical Wnt signal transduction pathway involving Dishevelled and Gsk3; the ability of the IGFBP domain to activate the reporter is inhibited by the dominant-negative Dishevelled construct dd1 (data not shown) (Sokol, 1996).

The above experiments address Wnt function during cleavage stages of development. At later stages, during gastrulation, Wnt signalling through the canonical pathway promotes ventrolateral fates. We found that dorsal injection of a plasmid expressing Xcyr61 caused a reduction in head and eye formation, suggesting that Xcyr61 can also activate Wnt signalling during gastrulation (data not shown). Thus, in two independent contexts, our results are consistent with the idea that Xcyr61 causes stimulation of Wnt signalling.

Cyr61 can also antagonise Wnt/β-catenin signalling

The results described above show that Xcyr61 has weak axis-inducing activity that is likely to occur through the Wnt signalling pathway. In an effort to elucidate the molecular basis of this phenomenon, we investigated whether Xcyr61 could act synergistically with Xwnt8 in such an assay. Surprisingly, instead of observing synergism between Xcyr61 and Xwnt8, we observed antagonism; Xcyr61, which alone induces partial secondary axis formation (Fig. 8C) inhibited secondary axis induction by Xwnt8 (Fig. 8A,B; Table 1). This inhibitory effect requires the TSP and CT domains (domains 3 and 4), since deletions that lacked these regions (construct 1,2) could not inhibit secondary axis formation (Fig. 8D), although they retained the ability to induce secondary axes (Fig. 8E). It is likely that the inhibitory activity resides in the CT domain, as does its ability to block secondary axis formation.

It is possible that Xcyr61 interferes with secondary axis formation by Xwnt8 by some indirect means, perhaps through its effects on cell adhesion. However, we find that Xcyr61 inhibits Xwnt8-induced activation of the TOPFLASH reporter in animal caps, which suggests that it interferes with Wnt/β-catenin signalling directly (Fig. 8H). This inhibitory activity of Xcyr61 requires the CT domain, as does its ability to block secondary
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Members of the Wnt family also signal through the so-called planar polarity pathway, which in vertebrate embryos is involved in the control of gastrulation. We investigated whether Xcyr61 can regulate the planar polarity pathway by asking whether it can prevent activin-induced elongation of Xenopus animal pole regions (Symes and Smith, 1987; Tada and Smith, 2000). Injection of full-length Xcyr61 mRNA inhibited such elongation (Fig. 9D). Although elongation of animal caps was inhibited in these experiments, the induction of mesodermal cell types, such as muscle-specific actin, was only slightly reduced (Fig. 9E-J). These results suggest that Xcyr61 also interferes with convergent extension, perhaps by reducing Wnt signalling through the planar polarity pathway, and that this inhibition requires the CT domain.

Xcyr61 can inhibit BMP signalling as well as modulate the Wnt pathway

The four modules of Cyr61 protein include the cysteine-rich (CR) VWC domain (domain 2; Fig. 1A). CR repeats are also present in other CCN family members, such as CTGF (Abreu et al., 2002), and also in chordin (Sasai et al., 1994) and short gastrulation (Francois and Bier, 1995). In these molecules the CR repeats bind to, and inhibit the action of, members of the BMP family (Abreu et al., 2002). We have tested Xcyr61 for anti-BMP activity by overexpression in animal pole explants. The doses of Xcyr61 RNA used in whole-embryo assays (100 pg to 1 ng) had little effect on animal caps but 4 ng Xcyr61 RNA induced cement gland formation and caused weak activation of NCAM (Fig. 10; data not shown), which suggests that Xcyr61 can, at least to some extent, inhibit BMP signalling. In further experiments, we found that co-expression of Xcyr61 and the truncated BMP receptor tBR induces additional heads, which is not observed with either construct alone (Fig. 10C-E). Because head induction requires the simultaneous inhibition of Wnt and BMP signalling, this suggests that the main function of Xcyr61 is to modulate Wnt signalling, rather than to inhibit the BMP pathway.

DISCUSSION

In this paper we describe the expression pattern of the Xenopus CCN family member Xcyr61 and show that it is involved in the
regulation of gastrulation. Both overexpression of Xcyr61 and the use of antisense morpholino oligonucleotides cause defects in morphogenesis, and, in this respect, Xcyr61 resembles other genes involved in gastrulation movements, where overexpression and inhibition can both cause disruption of gastrulation (Tada and Smith, 2000). As we discuss below, some of the effects of Xcyr61 on gastrulation may derive from its ability to support assembly of a fibronectin-rich extracellular matrix and to regulate cell-cell and cell-matrix adhesion. Other effects may be caused by its ability to modulate Wnt signalling; intriguingly, this secreted CR protein can both stimulate and inhibit Wnt signalling in a context-dependent manner.

**Adhesive properties of Xcyr61**

Although we have been unable to detect endogenous Xcyr61 mRNA during gastrulation, it is likely that Xcyr61 protein accumulates during cleavage stages. Results obtained in tissue culture indicate that once incorporated into the extracellular matrix, Cyr61 is very stable, with a half-life exceeding 24 hours (Yang and Lau, 1991). Experiments involving misexpression of the mouse gene suggest that the Xenopus gene product accumulates in the blastocoel roof (Fig. 1G,H), and in this respect Xcyr61 would resemble fibronectin, another gene product that is expressed throughout the early embryo (Lee et al., 1984). Like fibronectin, Cyr61, through its CT domain, supports the adhesion of Xenopus blastomeres (Fig. 6B). The morphology of cells plated on Cyr61 differs from that of cells adherent to fibronectin, and it seems likely that the behaviour of blastomeres adherent to the two substrates would differ, with those attached to fibronectin being more motile than those attached to Cyr61. Migratory behaviour during gastrulation might therefore depend on the levels of the two molecules in the extracellular matrix of the blastocoel roof.

However, it is likely that the influence of Xcyr61 on the extracellular matrix and on gastrulation is more profound than this because interference with Xcyr61 synthesis disrupts...
expression of Siamois axes in Xenopus

of Xcyr61 causes the formation of (usually partial) secondary pathway is provided by experiments in which overexpression evidence that Xcyr61 affects the canonical Wnt signalling through modulation of the Wnt signalling pathway. Direct Xcyr61 can both activate and inhibit Wnt signalling adhesion. A re-aggregation assay (Fig. 6I,J) shows that depletion of Xcyr61 from the embryo compromises Ca\(^{2+}\)-induced cell adhesion. Together, these data suggest that Xcyr61 plays a role in gastrulation through its own ability to support cell-matrix adhesion, through its role in the assembly of the extracellular matrix and through its influence on cell-cell adhesion.

**Xcyr61 can both activate and inhibit Wnt signalling**

Another way in which Xcyr61 might affect gastrulation is through modulation of the Wnt signalling pathway. Direct evidence that Xcyr61 affects the canonical Wnt signalling pathway is provided by experiments in which overexpression of Xcyr61 causes the formation of (usually partial) secondary axes in *Xenopus* embryos (Fig. 7A-F). It also induces the expression of Siamois (Fig. 7G) and cardiac actin (Fig. 7H-K) in isolated ventral marginal zone tissue, and activates expression of the TOPFLASH reporter in these cells (Fig. 7L). This ability of Xcyr61 to activate the Wnt pathway, which is weak compared with that of Xwnt8 (Fig. 7G) or Dishevelled (Fig. 7L), is likely to be mediated by the IGFBP domain (domain 1) (Fig. 7L).

However, to our surprise Xcyr61 also proved to be capable of inhibiting Wnt signalling. Thus, Xcyr61 prevented the formation of secondary axes in response to Xwnt8 (Fig. 8A-G), and, not only did it fail to activate the TOPFLASH reporter in animal caps, it inhibited its activation by Xwnt8 (Fig. 8H). Our experiments suggest that this inhibition is mediated by the CT domain (domain 4): this region of the protein is capable, alone, of preventing the formation of secondary axes (Fig. 8F) and it is required for inhibition of TOPFLASH activation (Fig. 8H). The ability of Xcyr61 to both activate and inhibit the Wnt pathway is discussed below. Together, these experiments are consistent with the suggestion that Xcyr61 inhibits the elongation of activin-treated animal caps by interfering with Wnt signalling. It is possible that the decrease in Xbra expression in embryos injected with Xcyr61 antisense morpholino oligonucleotides (Fig. 4D) is caused, in part, by the downregulation of Wnt signalling (Arnold et al., 2000; Yamaguchi et al., 1999), and this may also contribute to the disruption of gastrulation (Beddington et al., 1992; Conlon and Smith, 1999).

**Xcyr61: a versatile modular molecule**

Together, our results indicate that Xcyr61 is a versatile molecule that probably plays several roles in early *Xenopus* development. It is involved in the assembly of the extracellular matrix, in cell-matrix and cell-cell adhesion, in the upregulation and inhibition of Wnt signalling, and (albeit weakly) in the inhibition of BMP signalling. Some of these activities can be ascribed to particular domains of the protein. Adhesion of blastomeres to Cyr61 requires the CT domain, for example, as does the inhibition of Wnt signalling, where this domain is sufficient to inhibit secondary axis signalling by Xwnt8 (Fig. 8F). By contrast, stimulation of Wnt signalling appears to be mediated by the IGFBP domain (domain 1).

The abilities of the CT domain to regulate cell adhesion and to inhibit Wnt signalling may be related. This domain is required for the adhesion of fibroblasts (Grzeszkiewicz et al., 2001) and of *Xenopus* blastomeres (Fig. 6D) to Cyr61, and it also mediates the interaction of Cyr61 with heparan sulphate proteoglycans (HSPGs) (Chen et al., 2000). Exogenous heparin blocks the adhesion of *Xenopus* cells to Cyr61 (Fig. 6H) and this is likely to occur as a result of competition with cell-associated HSPGs for sites on Cyr61. HSPGs have also been implicated in the regulation of Wnt signalling (Topczewski et al., 2001; Tsuda et al., 1999), and it is possible that the ability of Xcyr61 to bind HSPGs is related to its ability to inhibit Wnt signalling. Another potential link between cell adhesion and the modulation of Wnt signalling by Xcyr61 might be provided by the integrins, which are the only known receptors for Cyr61 (Bökel and Brown, 2002; Grzeszkiewicz et al., 2001; Lau and Lam, 1999). Integrin-mediated adhesion of cells to the extracellular matrix recruits Dishevelled to the plasma membrane (Marsden and DeSimone, 2001), and this may enhance the ability of cells to respond to a Wnt signal.

Activation of Wnt signalling by Xcyr61 can occur through the IGFBP domain (domain 1). We do not know if the Wnt-stimulating activity of domain 1 of Xcyr61 is related to its putative IGF binding activity, although we note that activation of the IGF receptor in *Xenopus* embryos inhibits the Wnt pathway (Pera et al., 2001; Richard-Parpaillon et al., 2002). It is possible that domain 1 of Xcyr61 binds endogenous members of the insulin-like growth factor family and relieves this inhibition.

It is intriguing that Xcyr61 can function both as an activator and as an inhibitor of Wnt signalling. These two activities can be observed in similar cellular contexts (the equatorial and vegetal regions of the *Xenopus* embryo) and so it is unlikely that the different activities are caused by the presence or absence of specific co-factors. Rather, our experiments suggest that Xcyr61 can act to elevate Wnt signalling when it is at a low level and inhibit it when the level is high. It might behave, in effect, as a Wnt ’buffer’. This may explain why, in many embryos, depletion of Xcyr61 has little effect on axis formation.

**Conclusion: the role of Xcyr61 in the *Xenopus* embryo**

Our results suggest that Xcyr61 is a multifunctional molecule that plays a key role in modulating and integrating many pathways and types of cell behaviour during Xenopus development. These include cell-cell and cell-matrix adhesion, the stimulation and repression of Wnt signalling, and the inhibition of BMP signalling. In view of this wide range of activities, it is not surprising that for gastrulation to proceed normally, the level of Xcyr61 needs to be precisely controlled. In the future, we plan to investigate each of these activities separately, by investigating in more detail the functions of different domains of the molecule, and by using antisense morpholino oligonucleotides to inhibit splicing of individual domains of the endogenous protein. We also plan to define the mechanism by which Xcyr61 modulates Wnt signalling.
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