INTRODUCTION

Most animals undergo gastrulation to establish germ layers and embryonic axes. The dynamic morphogenetic cell movements that take place during gastrulation are highly coordinated. In Xenopus embryos, these cell movements, which include involution and convergent extension, are driven predominantly by mesodermal cells (Keller, 1991; Keller et al., 1992). This is particularly obvious during convergent extension, when polarized axial mesodermal cells intercalate in radial and mediolateral directions to cause dramatic elongation of the dorsal marginal zone along the anteroposterior (AP) axis (Shih and Keller, 1992; Wilson and Keller, 1991). However, the molecular mechanisms by which mesodermal cells become polarized and drive these movements are poorly understood.

Recently, it has been reported that a non-canonical Wnt signaling cascade (PCP pathway) has been shown to regulate convergent extension movements in Xenopus and zebrafish. Heparan sulfate proteoglycans (HSPGs) are known as modulators of intercellular signaling, and are required for gastrulation movements in vertebrates. However, the function of HSPGs is poorly understood. We analyze the function of Xenopus glypican 4 (Xg4), which is a member of membrane-associated HSPG family. In situ hybridization revealed that Xg4 is expressed in the dorsal mesoderm and ectoderm during gastrulation. Reducing the levels of Xg4 inhibits cell-membrane accumulation of Dishevelled (Dsh), which is a transducer of the Wnt signaling cascade, and thereby disturbs cell movements during gastrulation. Rescue analysis with different Dsh mutants and Wnt11 demonstrated that Xg4 functions in the non-canonical Wnt/PCP pathway, but not in the canonical Wnt/β-catenin pathway, to regulate gastrulation movements. We also provide evidence that the Xg4 protein physically binds Wnt ligands. Therefore, our results suggest that Xg4 functions as positive regulator in non-canonical Wnt/PCP signaling during gastrulation.

SUMMARY

Coordinated morphogenetic cell movements during gastrulation are crucial for establishing embryonic axes in animals. Most recently, the non-canonical Wnt signaling cascade (PCP pathway) has been shown to regulate convergent extension movements in Xenopus and zebrafish. Heparan sulfate proteoglycans (HSPGs) are known as modulators of intercellular signaling, and are required for gastrulation movements in vertebrates. However, the function of HSPGs is poorly understood. We analyze the function of Xenopus glypican 4 (Xg4), which is a member of membrane-associated HSPG family. In situ hybridization revealed that Xg4 is expressed in the dorsal mesoderm and ectoderm during gastrulation. Reducing the levels of Xg4 inhibits cell-membrane accumulation of Dishevelled (Dsh), which is a transducer of the Wnt signaling cascade, and thereby disturbs cell movements during gastrulation. Rescue analysis with different Dsh mutants and Wnt11 demonstrated that Xg4 functions in the non-canonical Wnt/PCP pathway, but not in the canonical Wnt/β-catenin pathway, to regulate gastrulation movements. We also provide evidence that the Xg4 protein physically binds Wnt ligands. Therefore, our results suggest that Xg4 functions as positive regulator in non-canonical Wnt/PCP signaling during gastrulation.

Key words: Heparan sulfate proteoglycan, Wnt signaling pathway, Gastrulation movements, Xenopus

INTRODUCTION

Most animals undergo gastrulation to establish germ layers and embryonic axes. The dynamic morphogenetic cell movements that take place during gastrulation are highly coordinated. In Xenopus embryos, these cell movements, which include involution and convergent extension, are driven predominantly by mesodermal cells (Keller, 1991; Keller et al., 1992). This is particularly obvious during convergent extension, when polarized axial mesodermal cells intercalate in radial and mediolateral directions to cause dramatic elongation of the dorsal marginal zone along the anteroposterior (AP) axis (Shih and Keller, 1992; Wilson and Keller, 1991). However, the molecular mechanisms by which mesodermal cells become polarized and drive these movements are poorly understood.

Recently, it has been reported that a non-canonical Wnt signaling cascade (PCP pathway) in Drosophila (Adler, 1992; Mlodzik, 1999), also participates in the regulation of convergent extension movements in Xenopus, as well as in the zebrafish embryo (Djiane et al., 2000; Heisenberg et al., 2000; Tada and Smith, 2000; Wallingford et al., 2000). The zebrafish silberblick (slb) locus encodes Wnt11, and Slb/Wnt11 activity is required for cells to undergo correct convergent extension movements during gastrulation. The signal transducer Dishevelled (Dsh) acts downstream of Slb/Wnt11 through domains specific to the non-canonical Wnt/PCP signaling cascade, and directly regulates cell polarity within cells undergoing convergent extension (Heisenberg et al., 2000; Tada and Smith, 2000; Wallingford et al., 2000). In addition, the relocalization of Dsh to the cell membrane is required for convergent extension movements in Xenopus gastrulae, in the same way as recruitment of Dsh to the membrane through the Frizzled (Fz) receptor is required for the PCP pathway in Drosophila (Axelrod, 2001; Wallingford et al., 2000). Although many genetic experiments have demonstrated downstream effectors of the non-canonical PCP Fz/Dsh signal in Drosophila (Shulman et al., 1998; Tree et al., 2002), and some of these effectors (e.g. JNK) function to regulate the convergent extension movements (Darken et al., 2002; Yamanaka et al., 2002), the precise signaling mechanism that regulates the convergent extension movements remains unclear.

In addition to the intracellular signaling mechanism, intercellular modulators are involved in regulating coordinated movements of large cell populations. Heparan sulfate proteoglycans (HSPGs) have been implicated in the modulation of intercellular signaling in vertebrates and in Drosophila, and have been shown to be required for gastrulation movements in the Xenopus embryo (Brickman and Gerhart, 1994; Itoh and Sokol, 1994). Glypican, a member of the membrane-associated
HSPG family, is known to regulate Wnt signaling in *Drosophila* (Baeg et al., 2001; Tsuda et al., 1999) and zebrafish embryos (Topczewski et al., 2001). However, the molecular function of the HSPGs is poorly understood. Therefore, we cloned a gene encoding *Xenopus* glypican 4 (Xgly4), a member of the HSPG family, and analyzed its role in the non-canonical Wnt/PCP signaling pathway during gastrulation. In situ hybridization revealed that Xgly4 is expressed in the dorsal mesoderm and ectoderm during gastrulation. Xgly4 overexpression and translational inhibition by a morpholino oligonucleotide inhibited the gastrulation movements of the embryo and the convergent extension (elongation) of activin-treated animal caps, but did not affect mesoderm induction. Rescue analysis with different Dsh mutants and Wnt11 demonstrated that Xgly4 functions in the non-canonical Wnt/PCP pathway, but not in the canonical Wnt/β-catenin pathway for convergent extension movements. Furthermore, we show that reducing the levels of Xgly4 inhibits the cell-membrane accumulation of Dsh, as does the inhibition of Wnt11 in the dorsal marginal zone. Finally, we provide evidence that the Xgly4 protein physically binds Wnt ligands.

**MATERIALS AND METHODS**

**Plasmid constructs and morpholino oligonucleotides**

The entire coding region of Xgly4, in clone 110i3 (pBS SK–) from the NIBB Mochii normalized *Xenopus* neurula library, was inserted into the EcoRI and Xho I sites of the CS2+ vector. For the immunoprecipitation assay, Xgly4-Flag, encoding amino acids 1-539 of Xgly4 with a Flag-tagged amino acid sequence at the C-terminal end, was used. We also used ACRD and ΔC, which lack amino acids 59-421 and 353-539 of the encoding region of Xgly4-Flag, respectively, and ΔGAG where the three putative glycosylated Ser (492, 496 and 498) residues of Xgly4-Flag were replaced with Ala residues. *Xenopus* activin (Thomsen et al., 1989), Xnr1 (Jones et al., 1995), XeFGF (a gift from Dr. H. Okamoto), Xwnt11 (Tada and Smith, 1995), Xdsh with Myc-tag (Sokol, 1996), Xdsh-ΔDIX (Wallingford et al., 2000) have been previously reported. Each construct was linearized, and capped mRNA was synthesized in vitro using the mMESSAGE mMACHINE SP6 kit (Ambion).

The Xgly4 morpholino sequence was 5'-TGCA TGGTGAGAAC-3'. We used the morpholino oligonucleotide 5'-CATAGATGTATAACGCTACGAAAG-3' for the ascidian gene HrTT-1 (Hotta et al., 1998) as a negative control (Gene Tools, LLC).

**Manipulation of embryos, microinjection of synthetic mRNA and morpholino oligonucleotide**

Unfertilized eggs were collected and fertilized in vitro as described previously (Suizki et al., 1997). Embryos were de-jellied using 3% cysteine and washed with water several times. Embryos were staged according to Nieuwkoop and Faber (Nieuwkoop and Faber, 1967). Synthesized RNA was injected into the animal pole or marginal zone of two- or four-cell-stage embryos, respectively, and the injected embryos were kept in 3% Ficoll/0.1x Steinberg’s solution as described (Yamamoto et al., 2000). The animal cap (AC) and dorsal marginal zone (DMZ) were dissected at stage 8 and stage 10.5, respectively, and cultured in 0.1% BSA/1x Steinberg’s solution (Asashima et al., 1990). For the standard *Xenopus* AC assay, ACs were cultured until stage 18, and scored for elongation.

**In situ hybridization**

Whole-mount in situ hybridization was performed with digoxigenin (DIG)-labeled probes, as described by Harland (Harland, 1991). Hybridization was detected with an alkaline phosphatase-coupled anti-DIG antibody and visualized by using BM purple (Roche Molecular Biochemicals). Antisense in situ probes against Xgly4 and Xwnt11 were generated by linearizing the pBS-KS-Xgly4 and pBS-KS-Xwnt11 constructs with NotI, and transcribing them with T7 RNA polymerase. Xbra (Smith et al., 1991), XmyoD (Hopwood et al., 1989), Xgsc (Blumberg et al., 1991; Cho et al., 1991), Xvent1 (Nishimatsu and Thomsen, 1998) and Xnot (von Dassow et al., 1993) have been reported previously.

**RT-PCR analysis**

Profiles of Xbra and Xwnt11 expression in the animal cap injected with each mRNA or morpholino oligonucleotide were examined semiquantitatively using RT-PCR (Yamamoto et al., 2000). ACs were collected when sibling embryos had developed to stage 11. The total RNA was then isolated using TRIzol (GIBCO/BRL), and cDNA was synthesized as previously reported (Yamamoto et al., 2000). PCR was performed with oligonucleotide primers: Xbra, 5’-GGATACATCTT-CTCAAGGCTTGAGA-3’ (upstream) and 5’-GTGTCGGCGTCCA-CAAAGTCCA-3’ (downstream); and Xwnt11, 5’-AAATGGCCACG-GAGTGTGGAG-3’ (upstream) and 5’-CCTGAGACTCTCTCAGTGG-GCC-3’ (downstream). The primer sequence of histone, an internal input control, was as previously described (Iemura et al., 1998).

**Northern blot analysis**

PolyA+ RNA was extracted from whole oocytes and staged embryos using TRIzol reagent (GIBCO/BRL) and oligo(dT) (Roche). Oocytes and embryos were staged according to Nieuwkoop and Faber (Nieuwkoop and Faber, 1967). RNA was run on a 1% agarose gel containing formaldehyde, blotted onto nylon membranes, and hybridized with a DNA probe in 50% formamide, 5xSSPE, 5% SDS and 100 μg/ml denatured salmon sperm DNA. The entire Xgly4 cDNA was used as the probe. Blots were hybridization overnight at 55°C and then washed twice in 0.1xSSPE, 0.1% SDS at 55°C for 1 hour each.

**Immunofluorescence**

The DMZ and AC were dissected, fixed in MEMFA and incubated with anti-Myc antibody (9E10, Santa Cruz Biotechnology; 1:200) for 16 hours at 4°C. Subsequently, these tissues were incubated with anti-mouse IgG goat serum, 1:200 for 2 hours at room temperature, and examined using fluorescence microscopy (Larabell et al., 1997). To quantitate the localization of Xdsh in cells, five cells were randomly chosen from the mRNA- or morpholino oligonucleotide-injected area, which was marked by fluorescence, and the intensity of Xdsh staining in the cytoplasm and membrane in each cell was quantitated by using NIH image. The closest four among five sets of data were processed for graphical presentation.

**Immunoprecipitation**

HEK293T cells were transiently transfected with the indicated constructs using the calcium phosphate method. Forty hours after transfection, the cells were lysed in lysis buffer (50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 5 mM EDTA, 0.5% NP-40, 50 mM NaF) in the presence of protease inhibitors. Immunoprecipitation was performed by incubating the extracts with the M2-Flag monoclonal antibody (Sigma) coupled to protein A Sepharose CL 4B at 4°C for 1 hour. The precipitates were then washed with lysis buffer and subjected to western blot analysis using an anti-HA antibody (Santa Cruz Biotechnology).

**RESULTS**

A novel *Xenopus* glypican is the ortholog of mammalian glypican 4

Proteoglicans have recently been shown to be a key
component in regulating the action of polypeptide growth factors in a number of developmental processes (Bernfield et al., 1999). To investigate the role of HSPGs during early *Xenopus* development, we searched our *Xenopus laevis* EST database (http://xenopus.nibb.ac.jp) for proteoglycan-related genes. We found a cDNA containing an open reading frame of 556 amino acids that displays 69% amino acid identity to human and mouse glypicans 4, and 57% identity to zebrafish knypek (*kny*; Fig. 1A). Thus, the new *Xenopus* glypican-related gene was named *Xenopus* glypican 4 (*Xgly4*). The deduced protein has a typical glypican structure, with an N-terminal signal peptide, an extracellular cysteine-rich domain and three putative O-glycosylation sites (Fig. 1B), which further supports the idea that Xgly4 is a member of the glypican family (Fig. 1A).

**Expression of Xglyp4 is temporally and spatially regulated**

As revealed by northern blotting, Xgly4 mRNA appears to be maternally expressed at low levels, and its transcripts of 7.3, 4.8, 3.0 and 2.5 kb were detected throughout early development. Interestingly, the two smallest transcripts were relatively abundant in gastrula embryos (Fig. 2A). The spatial expression pattern produced by whole-mount in situ hybridization showed that Xgly4 was preferentially expressed in the animal hemisphere at the cleavage stages of the early blastula (data not shown) and that its expression was later confined to the deep layer of dorsal ectoderm and mesoderm of gastrula stage 11 embryos (Fig. 2B; upper left and right). In later embryogenesis, Xgly4 was expressed along the neuroectoderm in the neurula, and then in the head and somites in tail bud embryos (Fig. 2B; bottom left and right).

The intriguing graded expression of Xgly4 along the dorsoventral (DV) axis of the early gastrula encouraged us to investigate how the expression was regulated. Consistent with the preferential dorsal expression in normal gastrulae, injection of mRNA for Xnr1, β-catenin or a dominant-negative type I BMP-receptor ALK3 (DN-BR) into the ventral blastomeres (to dorsalize embryo) induced ectopic expression of Xgly4 in the ventral region (Fig. 3B-D). In contrast to its expression in these dorsalized embryos, the expression of Xgly4 was diminished in ventralized embryos that had received an injection of Bmp4 mRNA in the dorsal region (Fig. 3F). These results suggest that, even if it is indirect, the expression of Xgly4 is positively regulated by the dorsalizing signal in DV axis formation.

**Loss-of-function and gain-of-function analyses of Xgly4 causes defective convergent extension movements**

To clarify the in vivo function of Xgly4, we performed loss-of-function and gain-of-function analyses. For the loss-of-
To analyze the functional homology of Xgly4 to kny, we injected Xgly4 mRNA into zebrafish kny-mutant embryos to examine whether Xgly4 could rescue the kny phenotype. The embryonic axis and organ primordia of kny mutant embryos are shorter along the AP axis and broader mediolaterally compared with their wild-type siblings (Topczewski et al., 2001). These phenotypes were significantly rescued by the injection of Xgly4 mRNA, which suggests that Xgly4 is a functional homolog of the zebrafish kny gene (Fig. 4D). Previous studies indicate that Xbra and Xwnt11, which are immediate-early targets of mesoderm inducers, are required for gastrulation movements (Tada and Smith, 2000). Therefore, we analyzed the expression of Xbra and Xwnt11 in embryos that had been injected dorsally with Xgly4Mo or wild-type Xgly4 mRNA. In situ hybridization analysis showed that Xbra and Xwnt11 were expressed normally (Fig. 4C). Using mesodermal (XmyoD, Xnot), ventral (Xvent-1), dorsal (Xgsc) and neural markers (Xen2), we found that embryos injected dorsally with Xgly4Mo exhibited defects in the positioning and morphology of the mesoendodermal and neural tissues, although they expressed normal levels of specific marker genes (Fig. 4C; data not shown). These results further suggest that both loss- and gain-of-function of Xgly4 perturb gastrulation cell movement without affecting mesodermal differentiation.

Convergent extension movements have been analyzed in a simple assay in which naive AC cells can elongate in response to activin; this is associated with the induction of cell populations that give rise to notochord and muscle (Smith and Howard, 1992). To analyze the effects of Xgly4 on activin-induced AC elongation, Xgly4 mRNA or Xgly4Mo was injected with activin mRNA into the animal pole and the AC assay was performed. Although tissue elongation occurred in ACs that had been injected with activin mRNA injection, the elongation in response to activin was strongly inhibited by coinjection of higher amounts of Xgly4 mRNA or Xgly4Mo. In addition, Xgly4Mo-induced inhibition was rescued with a low dose of wild-type Xgly4 mRNA without the native 5' UTR region (Fig. 5A). For the analysis of mesoderm induction by activin, the expression levels of both Xbra and Xwnt11 were measured using RT-PCR. The expression of these genes was not affected by the co-injection of Xgly4 mRNA or Xgly4Mo (Fig. 5B), which suggests that Xgly4 controls cell movements without regulating the transcription of mesodermal genes (mesoderm induction).

**Xgly4 regulates the non-canonical Wnt/PCP pathway during gastrulation**

The *Drosophila* proteins Dally and Dally-like (Dlp), which are *Drosophila* members of the glypican family, modulate the Wingless (Wg) pathway, regulating Wg activity extracellularly (Baeg et al., 2001; Lin and Perrimon, 1999; Tsuda et al., 1999). Therefore, we examined the possible function of Xgly4 in the Wnt pathway in *Xenopus*. In situ hybridization analysis revealed that Xgly4 transcripts were co-expressed with Xwnt11 at the DMZ and Xwnt5A at the dorsal ectoderm, but were not co-expressed with Xwnt8 (Christian et al., 1991; Ku and Melton, 1993; Moon et al., 1993; Tada and Smith, 2000). Several studies have shown that Xwnt5A and Xwnt11, but not Xwnt8, have dominant effects on gastrulation movements (Hoppler et al., 1996; Kelly et al., 1995; Moon et al., 1993; Tada and Smith, 2000). Therefore, Xgly4 could be involved in...
the regulation of the Wnt5A/Wnt11 pathway, which is also called the non-canonical Wnt/PCP pathway. To test whether Xgly4 functions in Xwnt11 signaling, the phenotype of embryos co-injected with Xgly4 and Xwnt11 mRNA was carefully examined. As shown in Fig. 6, embryos that had been injected with wild-type Xwnt11 mRNA showed disrupted development, including aberrations in gastrulation, neural tube closure and head formation, all of which are considered to arise from defects in gastrulation cell movements. These phenotypes were classified according to four grades of severity, and the embryos were scored using this index. Wild-type Xgly4 mRNA per se had a weak inhibitory effect on gastrulation. Interestingly, however, whereas co-injection of a low dose (0.5 pg) of Xgly4 mRNA with Xwnt11 mRNA enhanced the defective gastrulation movements caused by the Xwnt11 mRNA, co-injection of a high dose (25 pg) of Xgly4 reduced the defective phenotype. This indicates that the Xgly4 protein acts as a positive modulator of the Xwnt11 ligand but inhibits Xwnt11 signaling at a high dose (Fig. 6), as does Kny in slb (zebrafish wnt11) signaling (Topczewski et al., 2001).

In Drosophila, several experiments and additional genetic data have led to the prediction that, in vivo, Dsh protein may localize to the membrane in response to Fz signaling through the PCP pathway (planar cell polarity/non-canonical Wnt-signaling cascade), but not in response to activation of the β-catenin-dependent Wg pathway (canonical Wnt/β-catenin pathway) (Axelrod, 2001; Axelrod et al., 1998). Consistent with this idea, in dorsal mesoderm cells of the Xenopus embryo, the Xenopus dishevelled (Xdsh) protein accumulates at the cell membrane in a manner that depends on endogenous PCP signaling and controls gastrulation movements (Wallington and Harland, 2001; Wallington et al., 2000) (Fig. 7A). To examine whether Xgly4 is involved in the regulation of this conserved PCP pathway, we tested whether disruption of Xgly4 function perturbed the Xdsh protein localization. As we expected, the injection of Xgly4Mo disturbed the membrane localization of Xdsh, as did injection of dominant-negative Xwnt11 mRNA (Fig. 7B,C), which suggests that Xgly4 function is required for Xdsh protein localization to the membrane. Overexpression of Xgly4 mRNA into the DMZ also inhibited the accumulation of Xdsh protein at the membrane (Fig. 7D). Staining of Xdsh associated with plasma membrane varied from cell to cell but it was clear that the membrane localization of Xdsh was inhibited in dnWnt11-, Xgly4Mo- or Xgly4mRNA-injected embryos compared with uninjected or HrTT-1 Mo-injected embryos (Fig. 7E). Taken together, these results suggest that both the gain- and loss-of-function of Xgly4 lead to dislocalization of Xdsh protein, and this is consistent with previous observations that an adequately tuned level of Wnt11 signaling is necessary for normal gastrulation movements (Djiane et al., 2000; Tada and Smith, 2000).
To analyze the pathway-specific contribution of Xgly4 to Wnt signaling, we tried to rescue the inhibition of activin-induced elongation of AC explants (top right) mimics the convergent extension movements seen during gastrulation. This elongation was inhibited by Xgly4Mo (41 ng; middle left). The inhibition was rescued by a low dose (25 pg) injection of Xgly4 (middle right). A higher dose (500 pg) of Xgly4 inhibited activin-induced elongation (bottom left), but a low dose (25 pg) did not (bottom right).

(B) Expression levels of Xbra, Xwnt11 and Histone (control) were analyzed by RT-PCR assay. The expression levels of Xbra and Xwnt11 were not affected in any of the explants shown in A.

Fig. 5. Both Xgly4Mo and wild-type overexpression inhibit convergent extension movements. (A) Activin (0.25 pg)-induced elongation of AC explants (top right) mimics the convergent extension movements seen during gastrulation. This elongation was inhibited by Xgly4Mo (41 ng; middle left). The inhibition was rescued by a low dose (25 pg) injection of Xgly4 (middle right). A higher dose (500 pg) of Xgly4 inhibited activin-induced elongation (bottom left), but a low dose (25 pg) did not (bottom right).

To analyze the pathway-specific contribution of Xgly4 to Wnt signaling, we tried to rescue the inhibition of activin-induced elongation of ACs that is caused by injection of Xgly4Mo with pathway-specific Xdsh mutants. The inhibition of elongation caused by Xgly4Mo (Fig. 4) was rescued by wild-type Xdsh (data not shown) or by a Xdsh mutant lacking the DIX, but not the DEP, domain (Wallingford et al., 2000).

Because the DIX domain of Xdsh protein is required for the canonical Wnt/β-catenin pathway and the DEP domain is required for the non-canonical Wnt/PCP pathway (Axelrod et al., 1998; Boutros and Mlodzik, 1999; Boutros et al., 1998; Tada and Smith, 2000; Wallingford et al., 2000), it was shown that Xgly4 would participate in activin-induced elongation movements through the non-canonical Wnt/PCP pathway. Our results support the idea that Xgly4 affects gastrulation movement through the non-canonical Wnt/PCP pathway upstream of Dsh.

Based on the results of these rescue experiments, it seems likely that Xgly4 plays a role as a positive modulator of Xwnt11. Consistent with this functional interaction, we found that the HA-tagged Wnt11 ligand co-immunoprecipitated with the extracellular domain of Xgly4 protein (Fig. 9A). These results suggest that the Xgly4 protein in extracellular matrices binds the Wnt11 ligand to modulate its action on the Frizzled7 (Fz7) receptor (Djiane et al., 2000). We also found that HA-
Glypican in convergent extension

Tagged Wnt5A and Wnt8 ligands co-immunoprecipitated with Xgly4 protein (Fig. 9A). Therefore, Xgly4 may also regulate the activity of Xwnt5A in vivo because these two molecules are co-localized during early Xenopus development. However, Xgly4 is not likely to affect Xwnt8 signaling in a dominant manner given that their expression patterns are complementary to each other (data not shown). We were surprised to find that Xgly4 also bound to a structurally unrelated growth factor activin (data not shown). This may suggest that Xgly4 has a rather broad range of binding specificity to soluble ligands. However, activin activity was not affected by Xgly4 because Xgly4 did not interfere with mesoderm induction in the AC elongation assay, as revealed by Xbra expression (Fig. 5). To ascertain functional significance of the binding to these ligands, we examined Xgly4 binding to its receptor(s). Interestingly, we found that Xgly4 showed a preferential binding to Fz7 rather than to activin type I (ALK4) or type II (ActRIIA) receptors (Fig. 9B). Therefore, we propose that functional interaction of Xgly4 with growth factors depends on the physical interaction of Xgly4 with receptors. This further suggests that one of molecular mechanisms of the ligand presentation involves the physical interaction of glypicans and ligand-binding signaling receptors. We next examined which domain is required for binding to Wnt11. To address this question, we constructed Xgly4-mutant cDNAs designed to encode Gly4 proteins lacking the C-terminal region (Gly4\textsuperscript{DC}) and the cystein-rich domain (Gly4\textsuperscript{ACRD}), and replaced the three putative glycosylated Ser residues (Gly4\textsuperscript{AGAG}) with Ala residues (Fig. 9C). We found that activin-induced elongation was strongly inhibited by the injection of Xgly4\textsuperscript{DC} or Gly4\textsuperscript{AGAG}, as it was by wild-type Gly4, but it was not inhibited by the injection of Xgly4\textsuperscript{ACRD} mRNA (Fig. 9C). Because we also found that HA-tagged Wnt11 ligand co-immunoprecipitated with Gly4\textsuperscript{DC}, Gly4 may interact with...
Wnt11 through the CRD domain. Together, these results suggest that CRD domain is essential for the ability of Xgly4 to produce convergent extension movements. It is interesting to note that the Wnt11 receptor Fz and co-receptor Gly4 share a common structural feature (CRD) for binding the ligand.

**DISCUSSION**

Like other polypeptide growth factors, the Wnt family proteins have essential roles in many developmental processes in vertebrates, including limb formation and posteriorization of the central nervous system. Recent studies have revealed that, depending on the ligands and receptors involved, the Wnt proteins signal through three distinct intracellular signaling pathways: the canonical Wnt/β-catenin pathway; the non-canonical Wnt/PCP pathway; and the non-canonical Dsh-independent Wnt/Ca\(^{2+}\) pathway. The canonical Wnt/β-catenin pathway is essential for DV axis formation and patterning, contributing to the establishment of Spemann’s organizer in *Xenopus*. The two non-canonical pathways, which are thought to be triggered by Wnt5A/Wnt11, are required for cell movement (‘convergent extension’) during not only *Xenopus* but also zebrafish gastrulation. In this report, we have demonstrated that Xgly4 acts as a modulator in the non-canonical Wnt/PCP pathway during *Xenopus* gastrulation.

It has been shown genetically that mutations of the *Drosophila* Dally and Dlp proteins, or the zebrafish Kny protein perturb normal patterning or cell movements controlled by Wg or Wnt, respectively. It has been proposed that the proteoglycans Dally, Dlp and Kny act as components of a receptor complex that serves as a coreceptor for Fz. In addition to genetic evidence, we have been able to show successfully that Xgly4 physically interacts with the Wnt11 ligand (Fig. 9A) and the Fz7 receptor (Fig. 9B), which activate the non-canonical Wnt/PCP pathway. These results suggest that Xgly4 may present Wnt ligands to Fz receptors, in the same way as the FGF low-affinity HSPG receptor does with FGF high-affinity signalling receptors (Pellegrini et al., 2000; Schlessinger et al., 1995). We also found that Xgly4 physically interacts with Wnt8. The question arising from these results is whether Xgly4 affects the Xwnt8 signaling pathway. In fact, the overexpression of Xgly4 inhibits the Xwnt8-induced second axis. However, because Otx2, the anterior marker gene, was expressed in Xgly4Mo-injected embryos (data not shown), head formation, except eye formation (Fig. 4B), was not affected by reducing Xgly4 levels using Xgly4Mo; this lack of affect on head formation is similar to that in the zebrafish kny mutant (Topczewski et al., 2001). The expression of *XmyoD* gene, which is regulated by Xwnt8, was also unperturbed (Fig. 4C), and we also found that Xgly4 transcripts were not co-
expressed with Xwnt8 in gastrula (Christian et al., 1991; Ku and Melton, 1993; Moon et al., 1993; Tada and Smith, 2000). In addition, given that Xwnt8 is an endogenous ligand of Xgły4 and that Xgły4 acts as a co-receptor for Wnt8, depletion of Xgły4 by morpholino oligonucleotides is expected to result in a loss of Wnt8 activity, and in anteriorization of embryo, because Wnt8 is considered to be an inhibitor of head formation. These results suggest that Xgły4 is not involved in the regulation of the Xwnt8 canonical pathway in vivo during Xenopus gastrulation, but that it inhibits Xwnt8 activity only when it is overexpressed beyond the physiological level. Nevertheless, as several HSPGs have been shown to act as modulators of the canonical Wg/Wnt pathway in different model systems (Alexander et al., 2000; Baeg et al., 2001; Tsuda et al., 1999), we cannot rule out the possibility that Xgły4 could potentially act as a modulator of the canonical Wnt/β-catenin pathway if it is co-expressed with the corresponding ligands.

Although embryos in which Xgły4 protein levels were reduced exhibited defects of the dorsal convergent movements, embryos with overexpressed Xgły4 also exhibited these defects. We found that overexpression of Xgły4 inhibited the non-canonical Wnt/PCP pathway in the same way as the Xgły4 form (Fig. 9A). This suggests that, at least in the AC, Xgły4 affects only the non-canonical Wnt/PCP pathway.

REFERENCES


