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Connective-tissue growth factor modulates WNT signalling and interacts with the WNT receptor complex

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Summary

Connective-tissue growth factor (CTGF) is a member of the CCN family of secreted proteins. CCN family members contain four characteristic domains and exhibit multiple activities: they associate with the extracellular matrix, they can mediate cell adhesion, cell migration and chemotaxis, and they can modulate the activities of peptide growth factors. Many of the effects of CTGF are thought to be mediated by binding to integrins, whereas others may be because of its recently identified ability to interact with BMP4 and TGFβ. We demonstrate, using *Xenopus* embryos, that CTGF also regulates signalling through the Wnt pathway, in accord with its ability to bind to the Wnt co-receptor LDL receptor-related protein 6 (LRP6). This interaction is likely to occur through the C-terminal (CT) domain of CTGF, which is distinct from the BMP- and TGFβ-interacting domain. Our results define new activities of CTGF and add to the variety of routes through which cells regulate growth factor activity in development, disease and tissue homeostasis.

Key words: *Xenopus*, CTGF, CCN family, Wnt signalling, LRP6

Introduction

The control of cell division, cell differentiation and morphogenesis during embryonic development requires the coordinated regulation of a variety of extracellular signals. In *Xenopus*, for example, head formation requires the suppression of nodal, BMP and WNT signalling and this is achieved in part by the secreted protein Cerberus (Piccolo et al., 1999). Connective tissue growth factor (CTGF) is a member of the CCN family of secreted proteins, of which CTGF itself, Cyr61 and Nov are the founder members (Bork, 1993; Moussad and Brigstock, 2000; Perbal, 2001). CCN family members contain four characteristic domains encoded by separate exons and, like Cerberus, they exhibit multiple activities: they associate with the extracellular matrix, they can mediate cell adhesion, cell migration and chemotaxis (Babic et al., 1999; Chen et al., 2001; Jedsadayanmata et al., 1999; Lau and Lam, 1999), and they can modulate the activities of peptide growth factors (Abreu et al., 2002; Inoki et al., 2002). Disruption of the *Ctgf* gene in the mouse embryo indicates that CTGF is required for the coordination of chondrogenesis and angiogenesis during the development of the skeleton (Ivkovic et al., 2003).

The four domains of CTGF resemble those found in other secreted proteins (Bork, 1993). Domain 1 exhibits homology with the N-terminal region of the low-molecular-weight insulin-like growth factor binding proteins (IGFBPs) and with Twisted gastrulation (Tsg), which modulates signalling by BMP family members (Chang et al., 2001; Mason et al., 1994; Oelgeschlager et al., 2000). Domain 2 includes a von Willebrand factor type C repeat (VWC), and also displays similarities with the cysteine repeats in the BMP antagonist Short gastrulation (Sog)/Chordin (Abreu et al., 2002; Sasai et al., 1994). Domain 3 has homology with the thrombospondin type 1 repeat superfamily of ECM associated proteins (Adams, 2001). Finally, domain 4, or the C-terminal (CT) domain, shows similarity to the C terminus of Slit, a protein involved in axon guidance and cell migration (Bork, 1993; Brose and Tessier-Lavigne, 2000; Rothberg et al., 1990). This domain contains a cystine knot structure, which is also present in growth factors including the TGFβ superfamily, platelet derived growth factor (PDGF) and nerve growth factors (NGFs). It is believed to mediate protein-protein interactions or dimerisation (Bork, 1993; Schlunegger and Grutter, 1993; Vitt et al., 2001). The same motif is found in Wise (WNT modulator in surface ectoderm), a recently identified modulator of WNT signalling (Itasaki et al., 2003). A comparison of the CT domains of CTGF and of Cyr61, Slit and Wise is shown in Fig. 1A.

Many of the effects of CTGF arise through its ability to bind integrins (Lau and Lam, 1999) or to modulate signalling by transforming growth factor type β (TGFβ) and bone morphogenetic proteins (BMPs) (Abreu et al., 2002), but it is likely that this multi-domain protein has other activities, and indeed it is known that CTGF binds to the low density lipoprotein receptor-related protein/α2-macroglobulin receptor (Segarini et al., 2001). We show, using *Xenopus* embryos, that CTGF regulates signalling through the WNT pathway,

probably because of its ability to bind to the WNT co-receptor LRP6. This interaction involves the CT domain of CTGF, which is distinct from the TGFβ and BMP-interacting domain. This observation defines new activities of CTGF and adds to the variety of routes through which cells might regulate growth factor activity in development, disease and tissue homeostasis.

Materials and methods

Embryo manipulations and RT-PCR

Embryos of *Xenopus laevis* were obtained as described (Smith, 1993). They were staged according to Nieuwkoop and Faber (Nieuwkoop and Faber, 1975) and cultured in 10% Normal Amphibian Medium (NAM) (Slack, 1984). Microinjections were performed in 75% NAM containing 4% Ficoll. For animal cap assays, embryos at the two-cell stage were injected into both blastomeres at the animal pole. Animal caps were dissected at late blastula stage 8-9 and cultured until the relevant stage. To examine the effects of CTGF on early development, embryos were injected with *Ctgf* RNA in the animal pole of one or both blastomeres. They were cultured in 75% NAM until the late blastula stage and subsequently in 10% NAM. When necessary, animal caps were treated with partially purified activin at a concentration of 8 units/ml (Cooke et al., 1987). Dorsal marginal zone regions were dissected at early gastrula stage 10 from embryos previously injected in the marginal zone with 1.0 or 2.5 ng *Ctgf* RNA. They were cultured until sibling embryos reached neurula stage 15.

Ctgf and *CtgfΔCT* RNA were transcribed from the plasmids pSP64T-CTGF and pSP64T-CTGFΔCT (see below). Plasmids were linearised with *Xba*I and transcribed with SP6 RNA polymerase. RNA encoding Xwnt8 or Dsh was transcribed as described (Christian et al., 1991; Sokol, 1996).

Reverse transcription-polymerase chain reaction (RT-PCR) analysis was performed as described (Wilson and Melton, 1994). Primers for *EF1α*, *Muscle-specific actin*, *N-CAM*, *Xnr3* and *Siamois* were as described (Domingos et al., 2001; Hemmati-Brivanlou and Melton, 1994). *Ctgf* primers were 5′ CTC CTC ACA GAA CCG CTA CC 3′ (upstream) and 5′ GGC TTG TTT TGT GCC AAT TT 3′ (downstream).

Antisense morpholino oligonucleotides

An antisense morpholino oligonucleotide with the sequence 5′ GTACAGCAGCAGATTAGTTCTCTTC 3′, designed to inhibit translation of *Xenopus* CTGF, was purchased from GeneTools.

Expression constructs

To create constructs for expression in *Xenopus* embryos, *Ctgf* was cloned by PCR from stage 25 *Xenopus* embryo cDNA using primers designed against the *Ctgf* open reading frame (GenBank accession number U43524). The upstream primer was 5′ GCT AGA TCT **ATG** TCT GCA GGA AAA GTG ACA GC 3′, which corresponds to the first ATG (bold) and includes a *Bgl*II site (underlined). The downstream primer was 5′ CAG TCT CGA GTG **CTA** TGT CTC CAT ACA TTT TCC G 3′, which includes a stop codon (bold) and an *Xho*I site (underlined). The resulting fragments were cloned into a modified form of pSP64T (Tada et al., 1998). An alternative version of pSP64T-CTGF, which included 18 extra amino acids at its C-terminus, was used in some experiments. Similar results were obtained with both constructs. *CtgfΔCT* was cloned using the same upstream primer and a downstream primer that included an *Xho*I site (underlined) and a stop codon (**bold**). Its sequence was 5′ G

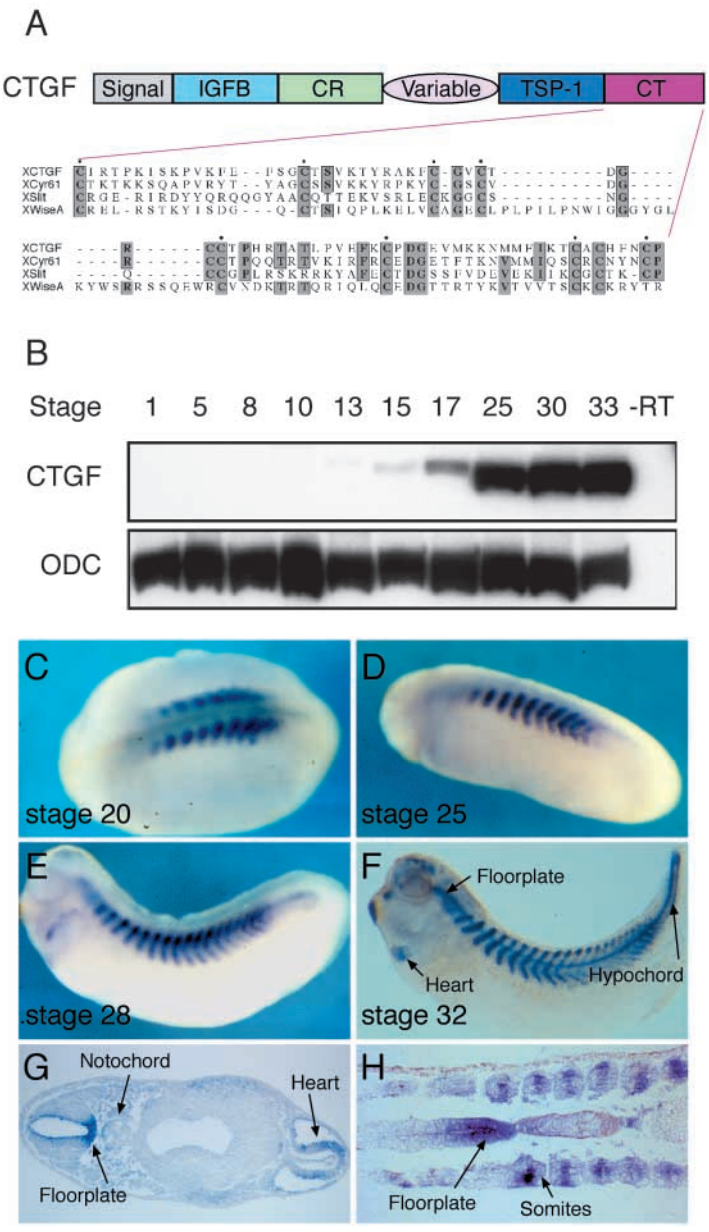

Fig. 1. CTGF and its expression pattern. (A) Schematic representation of the domain structure of CTGF. Shown below is an alignment of the CT domains of xCTGF, XCyr61, XSlit and XWiseA. The eight conserved cysteines are indicated by a dot and amino acids conserved in at least three out of the four proteins are shaded grey. The CT domain of xCTGF shows 52% amino acid identity with XCyr61, 27% with Xslit, and 19% with XWiseA. (B) Temporal expression pattern of *Ctgf* studied by reverse transcription-polymerase chain reaction. Stages are according to Nieuwkoop and Faber (Nieuwkoop and Faber, 1975). Ornithine decarboxylase (ODC) acts as a loading control. Note the absence of maternal expression of CTGF. (C-H) In situ hybridisation analysis of CTGF expression. (C) Stage 20. Expression is detectable in the somites and axial midline. (D) Stage 25. Expression persists in somites and axial midline. (E) Stage 28. Expression is detectable in brain and branchial arches. (F) Stage 32. CTGF is expressed in the heart, in distinct domains in the brain, in the floorplate and in the hypochord. (G,H) Sections through a stage 32 embryo confirm expression of *Ctgf* in floorplate, heart and somites. CT, C-terminal cystine knot; IGFB, Insulin growth factor binding domain; Signal, signal peptide; TSP-1, thromospondin type 1 repeat; CR, Von Willebrand type C repeat.

GGC CTC GAG **TTA** TTC ACA GGG CCT GAC CAT GC 3′. The resulting fragment was cloned into pSP64T (Tada et al., 1998) to create pSP64T-CTGFΔCT.

For expression of constructs in mammalian cells, IgG (Hsieh et al., 1999b), Frizzled8CRD-IgG (Hsieh et al., 1999b), LRP6N-IgG (Tamai et al., 2000) and DKK1-Flag (Mao et al., 2001) were as described. LRP6NΔ1,2-IgG and LRP6NΔ3,4-IgG, which lack EGF repeats 1 and 2 or 3 and 4, respectively, were generated by PCR as described previously (Itasaki et al., 2003; Mao et al., 2001). For expression in S2 insect cells, *Ctgf* and *CtgfΔCT* cDNAs were cloned into pMT/V5-HisB (Invitrogen) and the proteins were HA tagged at their C-termini, creating pMT/CTGF-HA and pMT/CTGFΔCT-HA.

In situ hybridization and X-gal staining

Embryos were fixed and processed for in situ hybridization essentially as described (Harland, 1991). Minor modifications included the use of BM purple (Boehringer Mannheim) as substrate. Sense and antisense *Ctgf* probes were made from a 700 base pair cDNA that includes the 3′ untranslated region. Other probes were *Xsox3* (Koyano et al., 1997), *N-tubulin* (Chitnis and Kintner, 1995), *Slug* (Mayor et al., 1995), *MLC1* (Theze et al., 1995), *Pax6* (Hirsch and Harris, 1997) and *Otx2* (Pannese et al., 1995).

For X-gal staining, fixed embryos were rinsed several times in PBS containing 0.1% Tween 20 (PBST), washed for 5 minutes in developing solution [7.2 mM Na_2HPO_4, 2.8 mM NaH_2PO_4, 150 mM NaCl, 0.1% Tween 20, 1 mM $MgCl_2$, 3 mM $K_3Fe(CN)_6$, 3 mM $K_4Fe(CN)_6$, pH 7.2] and transferred to fresh developing solution containing 0.027% X-gal for approximately 20 minutes at room temperature until adequate blue staining was achieved. They were then washed in PBST and stored in 100% methanol at –20°C until processed for in situ hybridisation.

Luciferase assays for TOPFLASH reporter activity

To measure the ability of CTGF and CTGFΔCT to inhibit Xwnt8 or Dishevelled activation of a TCF-dependent reporter construct, the TOPFLASH reporter plasmid (Korinek et al., 1998) was injected into *Xenopus* embryos together with pRLTK (Promega) as a reference plasmid, and with RNA encoding Xwnt8 or Dishevelled, either alone or in combination with RNA encoding CTGF or CTGFΔCT.

Luciferase activity was detected using the Promega Dual Luciferase Kit. Twenty animal caps were lysed in 200 μl of Passive Lysis Buffer (Promega) and 5 μl was assayed for luminescence.

Immunoprecipitation

S2 cells were grown as described in the *Drosophila* Expression System protocol (Invitrogen) and HEK293 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% foetal calf serum. Conditioned medium containing CTGF-HA or CTGFΔCT-HA was produced in S2 cells transiently transfected by the calcium phosphate method with pMT/CTGF-HA or pMT/CTGFΔCT-HA. One day after transfection, cells were transferred to serum-free medium (Invitrogen) and expression of CTGF-HA and CTGFΔCT-HA was induced by addition of copper sulphate to a final concentration of 500 μM. Medium was collected two days after induction and centrifuged at 20,000 ***g*** at 4°C for 30 minutes. Conditioned medium containing Frizzled8CRD-IgG, LRP6N-IgG, LRP6NΔ1,2-IgG, LRP6NΔ3,4-IgG or IgG was obtained from HEK293 cells (Hsieh et al., 1999b; Tamai et al., 2000) transiently transfected with the appropriate constructs using the Superfect transfection reagent (Qiagen) as described in the manufacturer's instructions. Cells were transferred to serum-free medium (Optimem, Gibco) one day after transfection and conditioned medium was collected 48 hours later.

Media were concentrated by ultrafiltration, combined as appropriate, and adjusted to the same volume by addition of control medium derived from untransfected cells. Mixtures were incubated overnight at 4°C and Protein A Sepharose beads were then added for 2 hours at 4°C. The beads were pelleted by centrifugation and, for low stringency experiments, were washed 5-6 times over 30 minutes in a buffer containing 150 mM NaCl, 50 mM Tris-HCl pH 7.5, 0.1% Triton X-100 and proteinase inhibitors. High stringency conditions involved an additional 3 washes in high stringency wash buffer (0.5 M NaCl, 50 mM Tris-HCl pH 7.5, 0.1% Triton X-100, 0.1% NP40, proteinase inhibitors). Unless otherwise indicated, experiments used high stringency conditions. Bound protein was eluted by boiling in SDS-sample buffer. Proteins were subjected to SDS-polyacrylamide gel electrophoresis and immunoblotted with the following antibodies according to the manufacturer's instructions: anti-IgG-HRP (Sigma), anti-HA-HRP (Roche) and anti-Flag-HRP (Sigma). The ECL+ detection technique (Amersham) was used for detection.

Results

Expression of CTGF

Ctgf transcripts in the *Xenopus* embryo are first detectable by the RT-PCR technique at the early neurula stage (Fig. 1B). Subsequently, expression is detected by in situ hybridisation in somites, floorplate, hypochord, olfactory placode and heart (Fig. 1C-H) (Abreu et al., 2002). Weak expression is also detectable in the pronephros (data not shown). Unlike Abreu and colleagues (Abreu et al., 2002), we have not been able to detect maternal expression of *Ctgf*, either by RT-PCR or by RNAase protection (Fig. 1B and data not shown).

Overexpression of CTGF mimics the effects of inhibiting components of the WNT signalling pathway

Injection into *Xenopus* embryos of an antisense morpholino oligonucleotide designed to inhibit translation of CTGF (see Materials and methods) caused no disruption of development (data not shown). A similar observation has been reported by Abreu and colleagues (Abreu et al., 2002). We note that mouse embryos in which the *Ctgf* gene is disrupted survive to birth and that mutant and wild-type embryos are indistinguishable at 12.5 days post-coitum (Ivkovic et al., 2003). This suggests that CTGF is essential only for later stages of development (see Discussion).

As an alternative approach to studying the function of CTGF in *Xenopus*, we overexpressed the protein by RNA injection at the one-cell stage. In 29% of embryos (n=55) this caused a disruption of gastrulation, whereas 56% displayed antero-posterior defects, with embryos forming a short trunk, enlarged cement gland, and reduced eyes (Fig. 2A-C). These defects were observed only rarely in embryos injected with the same amount of *lacZ* RNA. To characterise this phenotype in more detail, RNA encoding *Xenopus* CTGF, together with *lacZ* RNA to act as a lineage marker, was injected into one cell of *Xenopus* embryos at the two-cell stage, and the expression of neural and mesodermal markers was examined at neurula stages, when endogenous *Ctgf* is first expressed. We found that expression of *Otx2*, an anterior neural marker for forebrain and midbrain (Pannese et al., 1995), was expanded posteriorly on the injected side (Fig. 2D). *Pax6*, which at this stage is expressed in the prospective forebrain and in two dorso-lateral stripes that will form parts of the hindbrain (Hirsch and Harris, 1997), showed slight expansion of the forebrain domain, whereas the future hindbrain expression domain was decreased (Fig. 2E). These data indicate that CTGF is able to expand the territory of anterior neural structures.

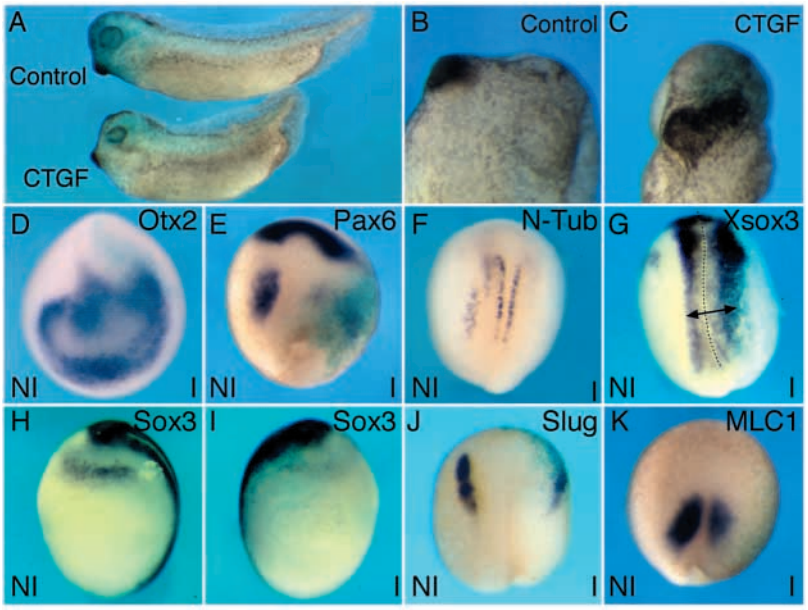

Fig. 2. The effects of overexpression of CTGF resemble those caused by inhibition of WNT signalling. (A) Overexpression of CTGF (bottom embryo) causes a shortening of *Xenopus* embryos. Three independent experiments were performed with similar results each time. (B,C) Compared with controls (B), CTGF-injected embryos have an enlarged cement gland (C). (D-K) Unilateral overexpression of CTGF causes changes in gene expression that resemble those caused by inhibition of WNT signalling. (D) The expression domain of *Otx2* is expanded. (E) CTGF causes a down-regulation of the future hindbrain domain of *Pax6* and slightly expands the anterior domain. (F) CTGF causes down-regulation of *N-tubulin* in the neural plate. (G) The expression domain of *Xsox3* in the neural plate is expanded. (H,I) Compared with the control side of the embryo (H), CTGF causes down-regulation of *XSox3* in the dorsolateral placode (I). (J) CTGF causes down-regulation of *Slug*. (K) The muscle-specific gene myosin light chain 1 (*MLC1*) is down-regulated by *Ctgf*. I: injected; NI: Not injected. The injected sides of embryos are identified by pale blue *lacZ* staining.

Overexpression of CTGF in the neural tube decreases numbers of terminally differentiated primary neurons, as judged by reduced expression of *N-tubulin* (Chitnis and Kintner, 1995) (Fig. 2F), and this is accompanied by an expansion of the domain of expression of *Xsox3*, a marker of undifferentiated neurons (Penzel et al., 1997; Zygar et al., 1998) (Fig. 2G). Together, these results indicate that widespread expression of CTGF causes an expansion of the neural plate and an inhibition or delay of primary neurogenesis. We also noted that although *Xsox3*-positive cells are increased in the neural plate, they are reduced in the dorsolateral placode (Fig. 2H,I). CTGF also affected the production of migrating neural crest cells as revealed by reduced *Slug* expression (Fig. 2J).

We next examined the effect of overexpression of CTGF on axial mesoderm; *Ctgf* is strongly expressed in somite tissue by the late neurula stage. The skeletal muscle-specific gene *myosin light chain 1* (*MLC1*) (Theze et al., 1995) is down-regulated by *Ctgf* (Fig. 2K). This will probably represent a delay in expression rather than a complete inhibition, because at later stages somites do form and appear relatively normal. Overall, the results described in Fig. 2 indicate that overexpression of CTGF anteriorises the neural tube, and inhibits or delays primary neurogenesis, neural crest formation and muscle development.

Some of the effects caused by overexpression of CTGF, such as the expansion of *Otx2* and *Xsox3*, and the occasional induction of partial secondary axes (Abreu et al., 2002) (data not shown), may be because of inhibition of BMP signalling. We note, however, that the actions of CTGF are also reminiscent of the effects observed upon inhibition of WNT signalling. For example, dominant-negative Xwnt8 (Hoppler et al., 1996) and the WNT antagonist Frzb (Leyns et al., 1997; Wang et al., 1997; Xu et al., 1998) all cause axial shortening and a decrease in somitic muscle. In neural crest formation, WNT1, WNT3A and positive components of the WNT signalling pathway such as Frizzled3, β-catenin and LRP6 all increase the production of neural crest cells (Wu et al., 2003). In contrast, inhibition of Frizzled3 function, like overexpression of CTGF (Fig. 2I), reduces *Slug* expression (Deardorff et al., 2001). Furthermore, overexpression of GSK3, a negative regulator of WNT signalling, causes enlargement of the cement gland and an impairment of eye formation (Itoh et al., 1995; Pierce and Kimelman, 1996), as does CTGF.

CTGF inhibits the effects of overexpression of XWNT8

To address more directly the question of whether CTGF affects WNT signalling, and in particular the canonical WNT signalling pathway, we first asked whether CTGF influences the induction of secondary axes in *Xenopus* embryos by Xwnt8 (Smith and Harland, 1991; Sokol et al., 1991). RNA encoding Xwnt8 was injected ventrally into *Xenopus* embryos at the 4- to 8-cell stage in the presence or absence of RNA encoding CTGF. CTGF inhibited secondary axis induction by *Xwnt8* (Fig. 3A-D) and also inhibited activation of the direct WNT targets *Siamois* and *Xnr3* (Brannon et al., 1997; McKendry et al., 1997) in ventral marginal zone tissue (Fig. 3E). Similarly, CTGF reduced activation of the TOPFLASH TCF reporter (Korinek et al., 1998) in *Xenopus* animal caps, suggesting that it interferes directly with the canonical WNT pathway (Fig. 3F).

CTGF and non-canonical WNT signalling

The above experiments indicate that CTGF can inhibit the canonical WNT signalling pathway involving β-catenin. We next asked whether CTGF can also interfere with β-catenin-independent WNT signalling. Non-canonical WNT signalling has been implicated in the regulation of gastrulation in vertebrate embryos, and it involves, at least in part, a mechanism resembling the *Drosophila* planar cell polarity (PCP) pathway. In *Xenopus* this pathway includes recently identified vertebrate homologues of *Drosophila* PCP genes such as Strabismus, as well as ligands such as WNT11, Frizzled receptors such as Fz7, and Dishevelled (Tada et al., 2002; Veeman et al., 2003a). Non-canonical WNT signalling

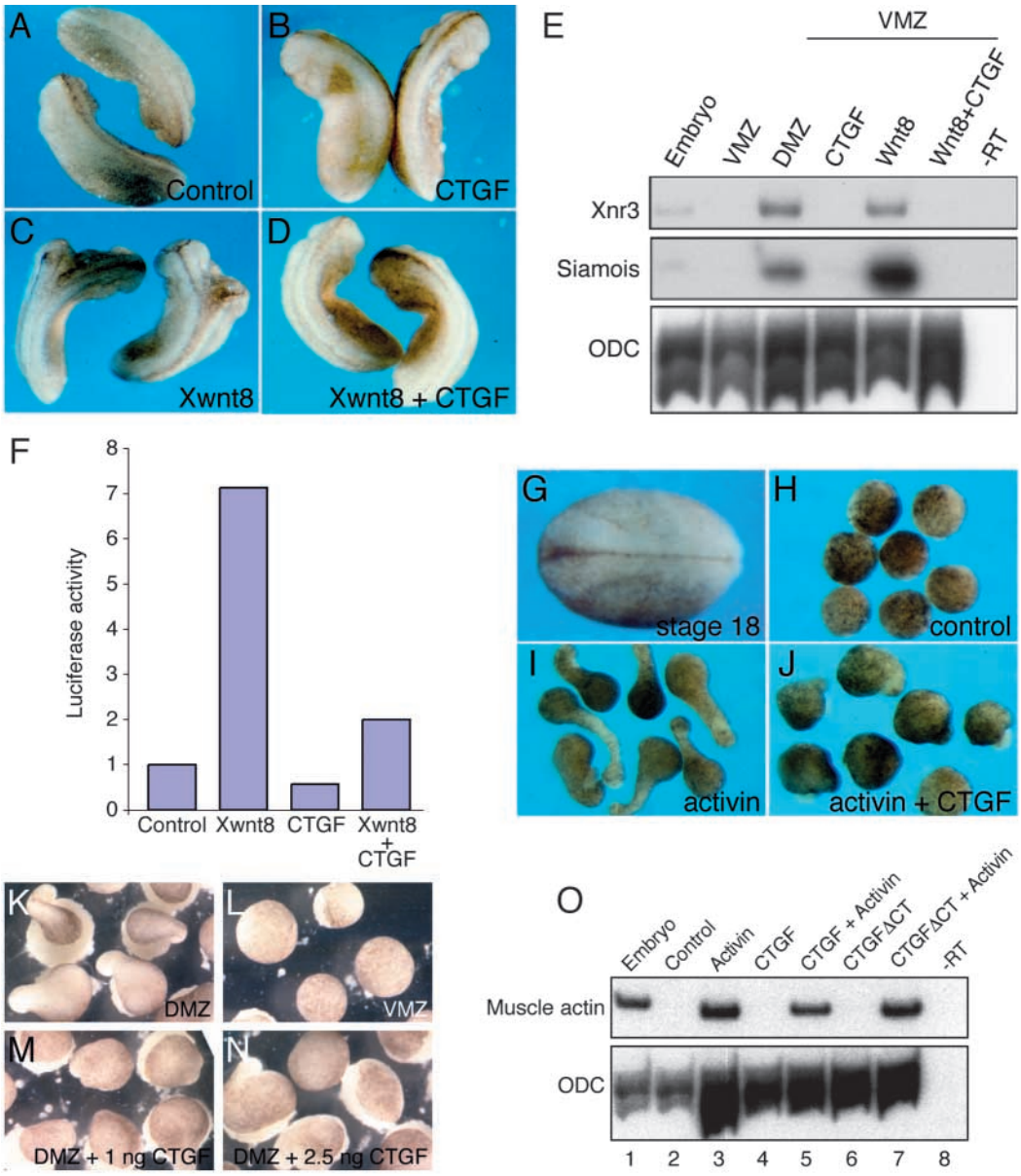

Fig. 3. CTGF inhibits WNT signalling. (A-D) Induction of duplicated axes by Xwnt8 is inhibited by CTGF. (A) Control embryos at stage 28. (B-D) Embryos were injected at the 4-8-cell stage into one ventral-vegetal blastomere with RNA encoding CTGF alone (B), Xwnt8 alone (C), or both (D). Secondary axis induction is inhibited by CTGF. (E) CTGF inhibits induction of *Xnr3* and *Siamois* by Xwnt8. Ventral marginal zone (VMZ) or dorsal marginal zone (DMZ) tissue was isolated from *Xenopus* embryos at early gastrula stage 10. Some embryos had previously been injected with RNA encoding Xwnt8 or CTGF or both, as indicated. Expression of *Xnr3*, *Siamois* or, as a loading control, *ornithine decarboxylase* (*ODC*) was assayed by RT-PCR. –RT: No reverse transcription control. (F) CTGF inhibits induction of the TOPFLASH reporter by Xwnt8. *Xenopus* embryos at the 2-cell-stage were injected into both blastomeres with 20 pg TOPFLASH (firefly luciferase) DNA, 10 pg pRLTK (Renilla luciferase) DNA and, where indicated, 1 ng CTGF RNA or 50 pg Xwnt8 RNA or a combination of the two. Animal caps were dissected at stage 8, and 20 caps per sample were assayed for Luciferase activities 3 hours later. Firefly luciferase activities were then normalised to Renilla activities. This experiment represents a typical result out of three independent experiments. (G-J) CTGF inhibits activin-induced elongation of animal caps. (G) Control embryo at stage 18. (H) Control animal caps at the equivalent of stage 18 remain spherical. (I) Activin-treated animal caps elongate. (J) Elongation is inhibited by overexpression of CTGF. (K-N) CTGF inhibits the elongation of isolated dorsal marginal zone regions. Dorsal marginal zone regions were isolated from control embryos (K) or from embryos injected with 1 ng (M) or 2.5 ng (N) *Ctgf* RNA. Ventral marginal zone regions (L) acted as controls. Explants were cultured to the equivalent of stage 15 and scored as described in Table 1. Note that CTGF causes a reduction in the elongation of dorsal marginal zone regions. (O) CTGF and CTGFΔCT do not inhibit activin-induced expression of muscle-specific actin in *Xenopus* animal caps. Expression of muscle-specific actin and ODC was assessed by RT-PCR. –RT: No reverse transcription control.

also regulates gastrulation through modulation of intracellular calcium release (Veeman et al., 2003b), and other mediators of non-canonical WNT signalling include heterotrimeric G proteins that are thought to act upstream of Dishevelled (Penzo-Mendez et al., 2003).

Activin treatment of animal caps derived from control embryos causes them to elongate, in a manner resembling the convergent extension movements of gastrulation and neurulation, whereas untreated caps remain spherical (Fig. 3H,I). Interference with the WNT/planar cell polarity pathway inhibits convergent extension (Tada and Smith, 2000), and indeed animal caps derived from embryos overexpressing CTGF do not elongate in response to activin (Fig. 3J) and the elongation of isolated dorsal marginal zone regions is also reduced (Fig. 3K-N; Table 1). Significantly, CTGF does not inhibit muscle differentiation in activin-treated animal caps, consistent with the idea that it affects the planar polarity pathway in this assay (Fig. 3O, lanes 1-5).

Together, these results suggest that CTGF can interfere with non-canonical WNT pathway as well as the canonical pathway.

Table 1. Inhibition of elongation of dorsal marginal zone tissue by CTGF

		Elongation		
	n	–	+	++
VMZ	17	17	0	0
DMZ	20	0	0	20
DMZ + 1 ng CTGF	15	4	8	3
DMZ + 2.5 ng CTGF	30	13	15	2

In this respect CTGF resembles WNT antagonists such as sFRP but differs from others such as DKK1, which does not block activin-induced elongation of animal caps (Semenov et al., 2001; Xu et al., 1998).

The ability of CTGF to modulate WNT signalling resides in the CT domain

The ability of CTGF to modulate BMP signalling resides in the second domain of the protein, which contains Chordin-like repeats (Abreu et al., 2002). We speculated that its ability to modulate WNT signalling resides in the fourth or CT domain. This region contains a cystine knot (Bork, 1993) and is a potential binding site for heparan sulphate proteoglycans (HSPGs) (Ball et al., 2003; Brigstock et al., 1997; Kireeva et al., 1997), which play a role in the regulation of WNT signalling (Baeg et al., 2001; Chen et al., 2000; Lin and Perrimon, 1999; Ohkawara et al., 2003; Tsuda et al., 1999). Consistent with this observation, we find that a deleted form of CTGF which lacks the CT domain (CTGFΔCT) (Fig. 4A) cannot interfere with Xwnt8-induced secondary axis formation (Fig. 4B-D) and was less effective in inhibiting Xwnt8-induced activation of the TOPFLASH reporter (Fig. 4E). It was also unable to inhibit activin-induced elongation of *Xenopus* animal caps (Fig. 4F-H). However, like the wild-type protein (Fig. 2J), CTGFΔCT was still capable of blocking expression of N-tubulin (data not shown), confirming that the truncated protein retains some activity and suggesting that regions of the protein other than the CT domain are involved in the regulation of primary neurogenesis. Like the wild-type protein (Fig. 3O, lanes 1-5), CTGFΔCT does not inhibit muscle differentiation in activin-treated animal caps (Fig. 3O, lanes 3, 6, 7).

CTGF interacts with the WNT receptor complex

CTGF is a secreted protein, and it seems probable that it inhibits WNT activity by interacting with extracellular components of the WNT signal transduction pathway. Consistent with this idea, we observe that CTGF cannot inhibit activation of the TOPFLASH reporter by Dsh, which acts downstream of the WNT receptor (Fig. 4E). WNT family members signal through complexes comprising members of the Frizzled family of seven transmembrane domain receptors together with the low-density lipoprotein receptor-related proteins LRP5 and LRP6 (Bafico et al., 2001; Mao et al., 2001; Tamai et al., 2000; Wodarz and Nusse, 1998). The known WNT antagonists Cerberus and WIF-1 (Hsieh et al., 1999a; Piccolo et al., 1999) and the secreted Frizzled-related protein (Kawano and Kypta, 2003) inhibit signalling by binding to WNT ligands. In contrast, the WNT antagonist DKK1 acts by binding to LRP6 (Mao et al., 2001; Semenov et al., 2001), as does Wise (Wnt modulator in surface ectoderm), which shows homology with the CT domain of CCN family members

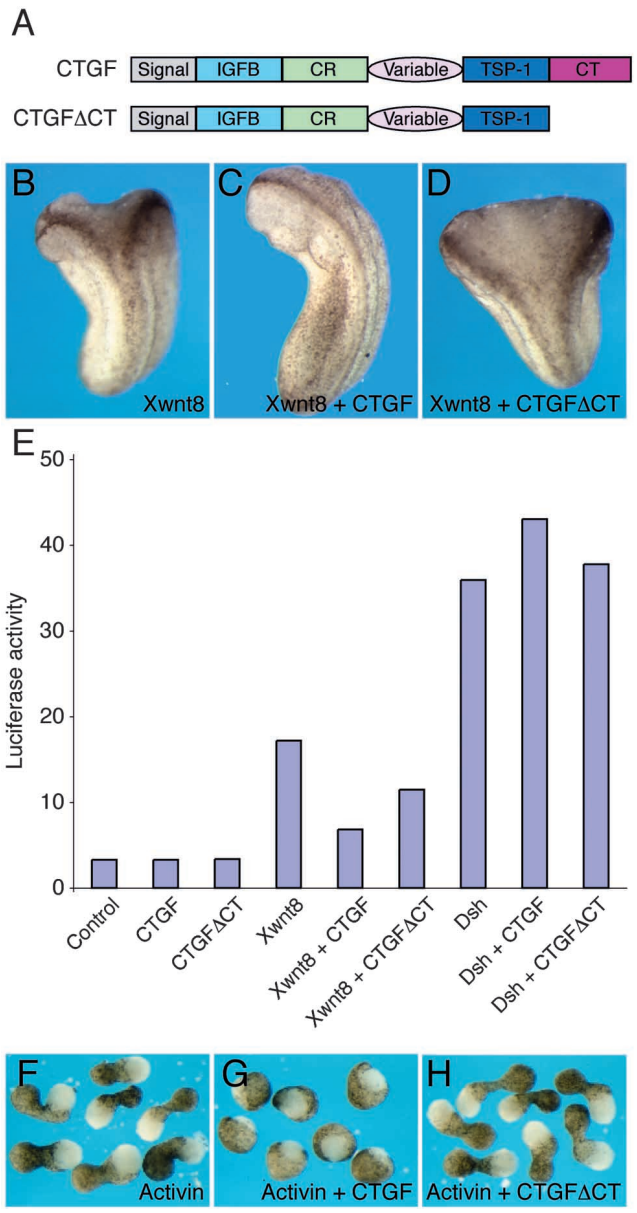

Fig. 4. Inhibition of WNT signalling requires the CT domain of CTGF. (A) Domain structures of CTGF and CTGFΔCT. (B-D) CTGFΔCT cannot inhibit induction of secondary axes by Xwnt8. (B) Secondary axis induced by Xwnt8. (C) Inhibition of secondary axis formation by CTGF. (D) CTGFΔCT cannot inhibit secondary axis formation. (E) CTGFΔCT is a poor inhibitor of Xwnt8-induced activation of the TOPFLASH reporter; CTGF cannot inhibit activation of the TOPFLASH reporter by Dishevelled. Both blastomeres of *Xenopus* embryos at the 2-cell stage received injections of 20 pg TOPFLASH DNA, 10 pg pRLTK DNA and the indicated combinations of 1 ng CTGF RNA, 1 ng CTGFΔCT RNA, 50 pg Xwnt8 RNA and 1 ng Dishevelled RNA. Animal caps were dissected at stage 8, and 20 caps per sample were assayed for Firefly and Renilla luciferase activities 3 hours later. Firefly luciferase activities were then normalised to Renilla activities. This experiment represents a typical result out of three independent experiments. (F-H) CTGFΔCT cannot inhibit activin-induced elongation of *Xenopus* animal caps. (F) Animal caps treated with 8 units/ml of activin protein undergo elongation. (G) Animal caps derived from embryos injected with 1 ng *Ctgf* RNA do not undergo elongation. (H) Elongation of animal caps is not inhibited by 1 ng of *CtgfΔCT* RNA.

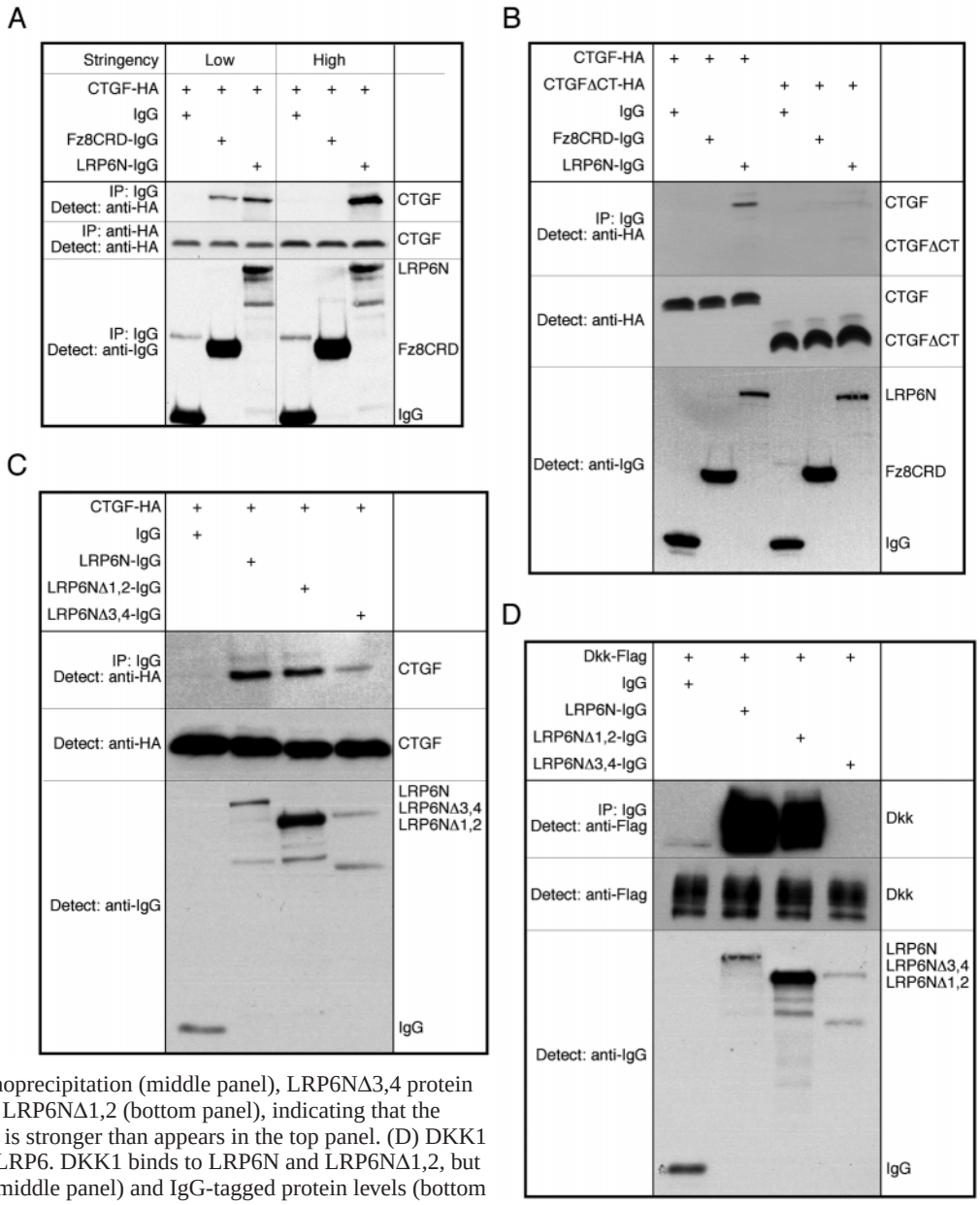

Fig. 5. CTGF interacts with the WNT receptor complex. In all experiments, proteins were provided in the form of conditioned medium, prepared as described in Materials and methods. Media were mixed as indicated, precipitated by Protein A Sepharose beads, and visualised by immunoblotting with the indicated antibodies. (A) CTGF interacts with LRP6 and Frizzled8 extracellular domains (LRP6N and FzCRD) under low stringency conditions (150 mM NaCl). Binding to LRP6, but not to Frizzled8, resists more stringent washing (500 mM NaCl) (top panel). Input levels of CTGF (middle panel) and the IgG-tagged proteins (bottom panel) are shown. (B) The CT domain is required for the interaction of CTGF with LRP6. CTGF but not CTGFΔCT is precipitated by LRP6N (top panel). Levels of CTGF and CTGFΔCT (middle panel) and of control IgG, Fz8CRD and LRP6N (bottom panel) prior to immunoprecipitation are shown. (C) CTGF binds to both EGF repeats 1, 2 and 3, 4 of LRP6. LRP6 extracellular domain (LRP6N) and two deletion constructs, LRP6NΔ1,2 and LRP6NΔ3,4, all interacted with CTGF whereas control IgG did not (top panel). Note that whereas CTGF protein levels are comparable in all lanes prior to immunoprecipitation (middle panel), LRP6NΔ3,4 protein levels are lower than both LRP6N and LRP6NΔ1,2 (bottom panel), indicating that the interaction of CTGF with this deletion is stronger than appears in the top panel. (D) DKK1 interacts with EGF repeats 3 and 4 of LRP6. DKK1 binds to LRP6N and LRP6NΔ1,2, but not to LRP6NΔ3,4 (top panel). Dkk- (middle panel) and IgG-tagged protein levels (bottom panel) prior to immunoprecipitation are shown.

(Itasaki et al., 2003) (Fig. 1A). The latter observation, together with previous work indicating that CTGF can bind to the related receptor LRP (Segarini et al., 2001), suggested that CTGF might function by interacting with the WNT receptor complex.

To ask whether CTGF can interact with Frizzled8 or LRP6, we combined conditioned medium containing HA-tagged CTGF with conditioned medium containing secreted IgG-tagged versions of LRP6 (LRP6N) or Frizzled8 (Frizzled8CRD). Co-immunoprecipitation experiments showed that CTGF can interact with the extracellular regions of both LRP6 and Frizzled8 under conditions of low stringency (150 mM NaCl), but that interaction with Frizzled8 is abolished when the precipitates were washed at high stringency (500 mM NaCl) (Fig. 5A). We conclude that CTGF can interact with LRP6 in solution and that this interaction is not disrupted by high salt washes. CTGF can also interact with Frizzled8, but this interaction is weaker and may require additional components. In support of this idea, we note that when the above constructs are co-transfected into HEK293 cells (rather than being analysed by mixing conditioned media), CTGF and Frizzled8 interact as strongly as CTGF and LRP6 (data not shown).

Further experiments demonstrated that CTGFΔCT is unable to bind LRP6 in this assay (Fig. 5B). This result is consistent with experiments described above indicating that the CT domain is required for complete inhibition of WNT signalling by CTGF (Fig. 4). We note that the CT domain is also required for interaction of CTGF with Frizzled8 (data not shown).

In an attempt to understand the molecular mechanism by which CTGF inhibits WNT signalling, we asked if it recognises the same domains of LRP6 as are recognised by

Xwnt8 or whether, like Dickkopf (Dkk), it interacts with distinct sites. The LRP6 extracellular domain contains four epidermal growth factor (EGF)-like repeats. Repeats 1 and 2 are required for interaction with WNT proteins, whereas Dkk interacts with EGF repeats 3 and 4 (Mao et al., 2001). Experiments involving immunoprecipitation of mixed conditioned media indicate that CTGF can interact both with EGF repeats 1 and 2 (LRP6NΔ3,4) and with EGF repeats 3 and 4 (LRP6N Δ1,2) (Fig. 5C). In the same assay DKK1 interacts specifically with the region containing EGF repeats 3 and 4 (Fig. 5D), as previously reported (Mao et al., 2001). Interestingly, co-transfection experiments indicate that CTGF binds preferentially to EGF repeats 1 and 2 (data not shown). The significance of the latter observation is not clear, but together our results indicate that the structural basis of the interaction of CTGF with LRP6 differs from that of DKK1, suggesting that the two molecules inhibit WNT signalling by different mechanisms.

Discussion

The data we describe indicate that CTGF can inhibit signalling through the WNT pathway. This will probably occur through its ability to interact with the WNT receptor complex, and in particular with LRP6. Our data add to the diversity of mechanisms through which WNT signalling can be regulated during development and they identify another role for CTGF as a coordinator of multiple signalling pathways.

Regulation of WNT and TGFβ signalling by CTGF

Some of the effects of overexpression of CTGF in the *Xenopus* embryo, such as the upregulation of *Otx2* and *Xsox3* and the induction of partial secondary axes (Abreu et al., 2002; data not shown), may be because of inhibition of BMP signalling. Others, however, such as the inhibition of neural crest cell migration, might be better explained by inhibition of the WNT pathway (Deardorff et al., 2001; Garcia-Castro et al., 2002) (Fig. 2 and see Results). Consistent with this idea, we observe that CTGF can inhibit the ability of Xwnt8 to induce secondary axes in the *Xenopus* embryo (Fig. 3A-D), that it can inhibit induction of the direct WNT targets *Siamois* and *Xnr3* (Fig. 3E), and that it can inhibit activation of the TOPFLASH reporter construct (Fig. 3F). CTGF will probably act extracellularly, because activation of the TOPFLASH reporter by Dsh is not prevented by CTGF (Fig. 4E), and indeed we have demonstrated that CTGF can interact stably with LRP6 and weakly with Frizzled8, two components of the WNT receptor complex (Fig. 5).

How might CTGF inhibit WNT signalling? One possibility is that CTGF, like Wise (Itasaki et al., 2003), competes with WNT family members for binding to LRP6. Preliminary results are consistent with this possibility, but interpretation of such experiments is complicated by the weak interaction of WNT8 with LRP6 in our assay conditions (data not shown). Other mechanisms include the idea that CTGF inhibits WNT signalling by inducing conformational changes within LRP6 that might displace Dkk or alter interactions with other components (Boyden et al., 2002; Liu et al., 2003), by binding to heparan sulphate proteoglycans, by binding to both LRP and LRP5/6 or by interacting with the WNT inhibitor Kremen (see below).

The ability of CTGF to regulate both TGFβ signalling (Abreu et al., 2002) and WNT signalling is reminiscent of the activity of Cerberus, which regulates BMP and WNT signalling through interactions with the respective ligands (Piccolo et al., 1999).

CTGF and other WNT inhibitors

Many secreted inhibitors of WNT signalling have been identified. Some, like Cerberus, WIF-1 and Frzb, function by binding to WNT ligands themselves and may thereby inhibit interaction between ligand and receptor (Bafico et al., 1999; Hsieh et al., 1999a; Leyns et al., 1997; Piccolo et al., 1999; Wang et al., 1997; Xu et al., 1998). In contrast, DKK1 functions by binding to LRP6 and preventing the formation of a complex comprising WNT, Frizzled and LRP6 (Semenov et al., 2001). DKK1 can also form a ternary complex with Kremen, its high affinity receptor, which induces rapid internalisation and removal of LRP6 from the cell surface (Davidson et al., 2002; Mao et al., 2002).

Although both CTGF and DKK1 bind to LRP6 to inhibit WNT signalling, there are some differences in the modes of action of these two antagonists. First, DKK1 binds preferentially to the region of LRP6 containing EGF repeats 3 and 4 (Mao et al., 2001), whereas CTGF can interact with both EGF repeats 1, 2 and 3, 4 (Fig. 5C,D). Second, DKK1 is specific for the canonical WNT pathway, which exerts its effect through the stabilization of β-catenin (Semenov et al., 2001), whereas CTGF also appears to affect the non-canonical WNT pathway, which regulates gastrulation movements (Fig. 3G-N). And finally, CTGF appears to be a much weaker antagonist of the WNT pathway than is DKK1; overexpression of DKK1 causes embryos to develop with extremely short trunks and enlarged heads and cement glands (Glinka et al., 1998), whereas comparable or higher doses of CTGF cause much milder anteriorised phenotypes.

Another member of the CCN family, Cyr61, also regulates WNT signalling (Latinkic et al., 2003), as does Wise, a novel CT domain protein (Itasaki et al., 2003), although these proteins can stimulate the pathway as well as antagonise it. These observations suggest that this class of cystine knot domain proteins may generally be involved in the modulation of WNT activity. Indeed, our experiments demonstrate that the interaction of CTGF with LRP6 requires the CT domain, and overexpression of just the CT domain of Cyr61, albeit at high levels, is sufficient to inhibit Xwnt8-induced secondary axis induction in *Xenopus* (Latinkic et al., 2003). The same will probably be true for the highly conserved CT domain of CTGF (Fig. 1A). We note that the CTGF CT domain plays a major role in binding to heparin and possibly to HSPGs (Ball et al., 2003; Brigstock et al., 1997). It is possible that HSPGs stabilise the binding of CTGF to Frizzled, because this interaction is strong in experiments involving co-transfection but weak in experiments in which conditioned media are combined (Fig. 5A).

CTGF and the non-canonical WNT pathway

CTGF differs from DKK1 in that it is able to inhibit convergent extension movements in animal caps. LRP6 signals exclusively through the canonical WNT pathway involving β-catenin (Semenov et al., 2001), so the ability of CTGF to modulate the WNT-mediated planar cell polarity pathway must occur

through another route, perhaps involving interaction with Frizzled receptors (such as Frizzled7) (Djiane et al., 2000), or through cooperation with HSPGs such as Glypican 4 (Ohkawara et al., 2003). The latter suggestion is supported by the observation that the ability of CTGF to block the elongation of activin-treated animal caps resides in the CT domain. An alternative possibility, however, is that CTGF modulates convergent extension through interactions with integrins. CTGF binds to integrin receptors (Lau and Lam, 1999), and integrins are involved in the recruitment of Dishevelled to the plasma membrane, a key step in the WNT planar cell polarity pathway (Marsden and DeSimone, 2001).

The function of CTGF

Disruption of the mouse *Ctgf* gene causes impaired chondrogenesis and angiogenesis (Ivkovic et al., 2003), but mutant embryos develop rather normally until mid-gestation stages. This result is consistent with the observation that antisense morpholino oligonucleotides cause no defect in early *Xenopus* embryos (Abreu et al., 2002) (data not shown). It is possible that another CCN family member, Cyr61, compensates for some aspects of loss of CTGF, because they are co-expressed in many tissues and have similar biochemical activities (Lau and Lam, 1999).

Finally, are any of the functions of CTGF in the embryo mediated through WNT signalling? We note that the expression patterns of *Ctgf* and *Lrp5* and *Lrp6* during *Xenopus* development are strikingly similar, suggesting that the interactions we observe in vitro may also be significant in vivo (Houston and Wylie, 2002). Other components of the WNT signalling pathways are also present in the *Ctgf* expression domain, including *Frizzled* family members (Borello et al., 1999; Deardorff and Klein, 1999) and, in the floorplate, *Wnt4* (McGrew et al., 1992; Ungar et al., 1995). It may also be significant that mouse embryos lacking *Lrp5*, like those deficient in *Ctgf*, display abnormalities in bone formation and angiogenesis (Gong et al., 2001; Kato et al., 2002; Little et al., 2002), suggesting that modulation of the WNT pathway is compromised in *Ctgf*$^{-/-}$ embryos.

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