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Functional analysis of the *Xenopus* frizzled 7 protein domains using chimeric receptors

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ABSTRACT Seven-transmembrane receptors of the frizzled family can interact with secreted Wnt ligands and transmit Wnt signals into the cell. Dependent on the ligand receptor combination, distinct Wnt pathways are activated. *Xenopus* frizzled 7 (Xfz7) and Xwnt-8b as well as Human frizzled 5 (Hfz5) and Xwnt-5a can act synergistically in the activation of Wnt/β-catenin target genes *siamois* (*Xsia*) and *nodal related 3* (*Xnr3*) and in the induction of ectopic axes in *Xenopus* embryos. In order to characterize the role of different protein domains of Xfz7 in Wnt/β-catenin signaling, chimeric Xfz7/Hfz5 receptors were generated in which the extracellular (N5-TC7) or the intracellular domains (NT7-C5) between Xfz7 and Hfz5 were exchanged. We present evidence that the extracellular domain of Xfz7 can interact with Xwnt-5a and that the intracellular C-terminus can transmit a Wnt/β-catenin signal. Despite these abilities, Xfz7 and Xwnt-5a do not act synergistically in the activation of Wnt/β-catenin targets. This implies that the interaction of a frizzled receptor with different ligands can result in distinct cellular responses.

KEY WORDS: *Xenopus, Wnt signaling, Xfz7, Hfz5, Chimeric receptor.*

Introduction

Wnt proteins are cysteine-rich glycoproteins that form a family of highly conserved secreted signaling molecules. These can be found throughout the animal kingdom and are implicated in a variety of developmental processes and oncogenesis (Cardigan and Nusse, 1997; Moon and Kimelman, 1998; Gradl *et al.*, 1999). Wnt genes are not functionally equivalent and may exert their diverse effects through activation of distinct intracellular signaling pathways. Wnt proteins can be broadly divided into two functional classes based on their activity in various assays. The Wnt-1/wg class of molecules (Xwnt-1, -2, -3a, -7b, -8, -8b) can induce secondary axis when ectopically expressed in the ventral marginal zone of four-cell stage *Xenopus* embryos (McMahon *et al.*, 1989; Landesman and Sokol, 1997; Wolda *et al.*, 1993; Sokol *et al.,* 1991; Cui *et al.*, 1995). This effect of Wnt proteins is thought to be mediated by a signaling cascade that involves β-catenin (Wnt/β-catenin pathway). Other members of the Wnt family (Xwnt-4, -5a, -11) do not posses the capacity to induce axis formation (Du *et al.*, 1995; Moon *et al.*, 1993). The members of this class are able to antagonize the axis-inducing effect of the Wnt-1/wg class in *Xenopus* embryos and Wnt-5a is able to reverse the transforming properties of Wnt-1 in C57MG cells (Olson and Gibo, 1998). This class of Wnt proteins activates a signaling cascade that involves modulation of intracellular Ca^{2+} (Wnt/Ca^{2+} pathway) (Miller *et al.*, 1999).

The Wnt/β-catenin pathway is activated when the Wnt protein binds to a receptor of frizzled family of seven-transmembrane proteins (Bhanot *et al.*, 1996; Yang-Snyder *et al.*, 1996). This ligand-receptor interaction leads to activation of the phosphoprotein dishevelled, which inhibits phosphorylation of β-catenin by glycogen synthase kinase-3β (GSK-3β). This leads to stabilization of β-catenin because underphosphorylated β-catenin escapes the ubiquitin-proteasome degradation pathway (Aberle *et al.*, 1997). Increased cytoplasmic levels of β-catenin results in the nuclear accumulation of this protein where it binds to HMG box transcription factors of the TCF/Lef–1 family (Brunner *et al.*, 1997), activating a number of target genes including Organizer-specific factors such *siamois* (*Xsia*), *twin* (*Xtwin,*), and *nodal related 3* (*Xnr3)* (Brannon *et al.*, 1997; Laurent *et al.*, 1997; McKendry *et al.*, 1997).

Among the frizzled proteins identified in *Xenopus,* only frizzled 7 (Xfz7) and frizzled 8 (Xfz8) are implicated in the early development of *Xenopus* (Medina *et al.*, 2000; Deardorff *et al.*, 1998; Itoh

Abbreviations used in this paper: RT-PCR, reverse transcription polymerase chain reaction.

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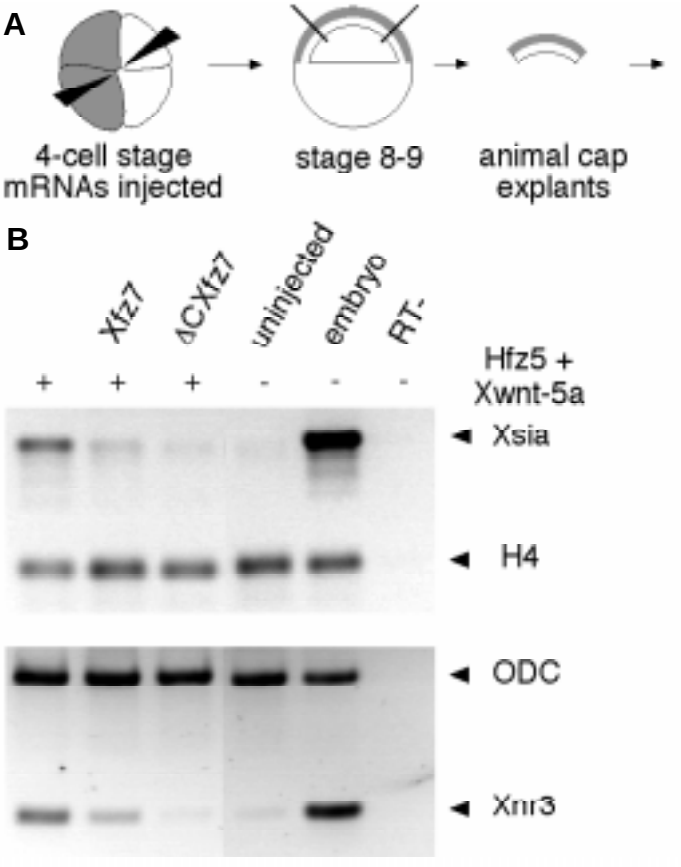

Fig. 1. Xfz7 can compete with Hfz5 for the Xwnt-5a ligand. (A) *Experimental scheme. Embryos were injected at the four-cell stage with 0.5 ng of Hfz5 and 0.02 ng of Xwnt-5a mRNA and in combination with 0.3 ng of Xfz7 or ΔCXfz7 mRNA. Animal caps were excised from embryos at stage 8.5 and the explants were cultured until uninjected control embryos reached stage 10. Xsia and Xnr3 expression was analyzed by RT-PCR.* **(B)** *Ornithine decarboxylase (ODC) or Histone H4 (H4) were used as PCR controls to ensure that an equal amount of RNA was used in each RT-PCR reaction. Xsia and Xnr3 transcription induced by Hfz5 and Xwnt-5a was strongly reduced when Xfz7 or ΔCXfz7 were coexpressed.*

et al., 1998). *Xfz7* mRNA is present in eggs and the zygotic transcription peaks at stage 10-11. Though the zygotic expression of *Xfz7* starts in the dorsal marginal zone, overexpression of *Xfz7* does not induce secondary axis in *Xenopus* embryos. Overexpression of *Xfz7* activates protein kinase C (PKC), inhibits morphogenetic movements and reduces adhesiveness to the C-cadherin matrix (Medina *et al.*, 2000). These results are similar to that of *Xwnt-5a* overexpression in *Xenopus* embryos, thus implicating its involvement in the Wnt/Ca^{2+} pathway. Xfz7 however, acts synergistically with Xwnt-8b in triggering the Wnt/β-catenin pathway. These results demonstrate that Xfz7 can act in both the Wnt/β-catenin and the Wnt/Ca^{2+} pathway, depending on the ligand (Medina *et al.,* 2000). *Xfz7* and *Hfz5* do not trigger the Wnt/β-catenin pathway when ectopically expressed in *Xenopus* embryos. Hfz5 but not Xfz7, in combination with Xwnt-5a, can trigger the Wnt/β-catenin pathway (He *et al.*, 1997; Medina *et al.*, 2000). It is crucial to understand how *Xenopus* frizzled receptors differentially activate the Wnt/β-catenin or Wnt/Ca^{2+} pathways. It is also important to understand how a specific frizzled molecule behaves in presence of different Wnt ligands in triggering either of the two Wnt signaling pathways. To address these questions, chimeric frizzled receptors were constructed by exchanging the homologous extracellular as well as the seven transmembrane and cytoplasmic domains of Xfz7 and Hfz5. These frizzled receptor chimeras were tested for their ability to activate the Wnt/β-catenin pathways by monitoring the transcriptional activation of target genes (*Xnr3* and *Xsia*) and by phenotypic analysis of *Xenopus* embryos expressing these chimeric receptors alone or in combination with *Xwnt-5a*.

Results

Xfz7 can interfere with Xwnt-5a/Hfz5 signaling

Activation of the Wnt/β-catenin pathway can be assayed by monitoring transcriptional activation of target genes *Xnr3* and *Xsia* in animal caps. These target genes can be activated in animal caps overexpressing the Wnt-1 class of molecules such as Xwnt-8. When Xwnt-5a and Hfz5 are combined, a Wnt-1-type signal is generated and *Xnr3* and *Xsia* are activated (He *et al.*, 1997). We therefore set out to investigate whether Xfz7 could interfere with the Hfz5/Xwnt-5a interaction in an animal cap assay. When *Xfz7* or *ΔCXfz7*, a mutant lacking the cytoplasmic domain, were coexpressed with *Hfz5* and *Xwnt-5a*, *Xnr3* and *Xsia* transcription were reduced compared to *Hfz5/Xwnt-5a*-injected animal caps (Fig. 1). This result can be interpreted as a competition between Hfz5 and Xfz7 for the Xwnt-5a ligand. The interaction of Xfz7 and Xwnt-5a, however, does not activate the Wnt/β-catenin pathway (Medina *et al.*, 2000). The observation that ΔCXfz7, a mutant impaired in signal transduction, reduces *Xnr3* and *Xsia* expression supports the notion that sequestration of Xwnt-5a rather than an intracellular mechanism inhibits the transcription of Wnt target genes in this assay.

Activities of chimeric Xfz7/Hfz5 receptors in Xenopus *animal caps*

In an attempt to characterize the role of the extracellular and the intracellular domains of Xfz7 in Wnt/β-catenin signaling, frizzled receptor chimeras were constructed by exchanging the homolo-

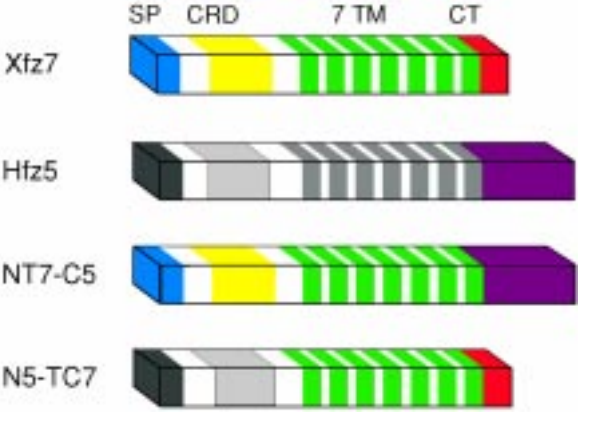

Fig. 2. Chimeric Xfz7/Hfz5 constructs. *Schematic domain structure of Xfz7 and Hfz5. For the chimeric receptor NT7-C5, the extracellular as well as the seven-transmembrane domains of Xfz7 and the cytoplasmic domain of Hfz5 were joined. The construct N5-TC7 contains the extracellular region of Hfz5 and the seven-transmembrane and cytoplasmic domains of Xfz7. For details, see Materials and Methods.*

gous regions from Xfz7 and Hfz5. The chimeric receptor NT7-C5 contains the extracellular and the seven-transmembrane domains of Xfz7 and the cytoplasmic domain of Hfz5 (Fig. 2). The chimera designated as N5-TC7 contains the extracellular region of Hfz5 and the seven-transmembrane as well as the cytoplasmic domains of Xfz7 (Fig. 2).

To test the activation of the Wnt/β-catenin pathway by the chimeric receptors, synthetic mRNA for these constructs (0.5 ng) was injected into the animal pole of four-cell stage embryos and the activation of *Xnr3* and *Xsia* was analyzed by RT-PCR. NT7-C5 and N5-TC7 did not activate the expression of *Xnr3* or *Xsia* (Figs. 3B, 4B). Next, the activation of the Wnt/β-catenin pathway by these chimeric receptors in combination with Xwnt-5a was tested in the same type of animal cap assays. NT7-C5 and N5-TC7 acted synergistically with Xwnt-5a and activated the expression of *Xnr3* and *Xsia* (Figs. 3B, 4B). This suggests that the chimeric receptors trigger the Wnt/β-catenin pathway in the presence of Xwnt-5a and fail to do so in absence of any exogenous Wnt ligand.

Ectopic expression of chimeric frizzled receptors in Xenopus *embryos*

Activators of the Wnt/β-catenin pathway can dorsalize *Xenopus* embryos when injected into the ventral marginal zone. To test the effect of the chimeric receptors on the development of *Xenopus* embryos, synthetic mRNA for *NT7-C5* and *N5-TC7* was injected into the ventral vegetal blastomeres at the four-cell stage and their phenotypic effect was scored at neurula and tailbud stages.

Overexpression of *NT7-C5* in embryos led to the formation of shortened tail and trunk structures (Fig. 3C). The formation of anterior structures such as eyes and cement glands was unaffected. Embryos coinjected with *NT7-C5* and *Xwnt-5a* mRNA formed partial secondary axes lacking head structures such as eyes and cement glands (Fig. 3D).

Embryos expressing *N5-TC7* developed normally, like their uninjected siblings (Fig. 4C), however when *N5-TC7* was injected in combination with *Xwnt-5a,* this chimeric receptor induced secondary axes without heads (Fig. 4D). As expected, embryos expressing *Hfz5* and *Xwnt-5a* developed secondary body axes (Fig. 4E). Histological analysis of the axis structures induced by NT7-C5, N5-TC7 and Hfz5 in combination with Xwnt-5a demonstrated the presence of ectopic notochords, neural tissue and somites (Figs. 3D′, 4D′,E′).

These results demonstrate that the chimeric receptors, NT7-C5, and N5-TC7 can act synergistically with Xwnt-5a in inducing secondary axes in *Xenopus* embryos. This is consistent with the results obtained with animal cap assays showing that *Xnr3* and *Xsia* can only be induced through the receptors in combination with Xwnt-5a.

Discussion

Activities of Xfz7 and Hfz5 in Xenopus *embryos*

It has been shown that *Xenopus* frizzled proteins can act as receptors in either Wnt/β-catenin or Wnt/Ca^{2+} pathways (Miller *et al.,* 1999). It is not understood, however, how frizzled receptors activate either of these pathways. It has been argued that the extracytoplasmic domains are involved in binding to different Wnt ligands and the transmembrane and the cytoplasmic domains are involved in triggering the Wnt/β-catenin or the Wnt/Ca^{2+} pathways. In such a scenario, only the frizzled receptors with a correct combination of extracytoplasmic and cytoplasmic domains should be able to trigger either of these two pathways. To test this

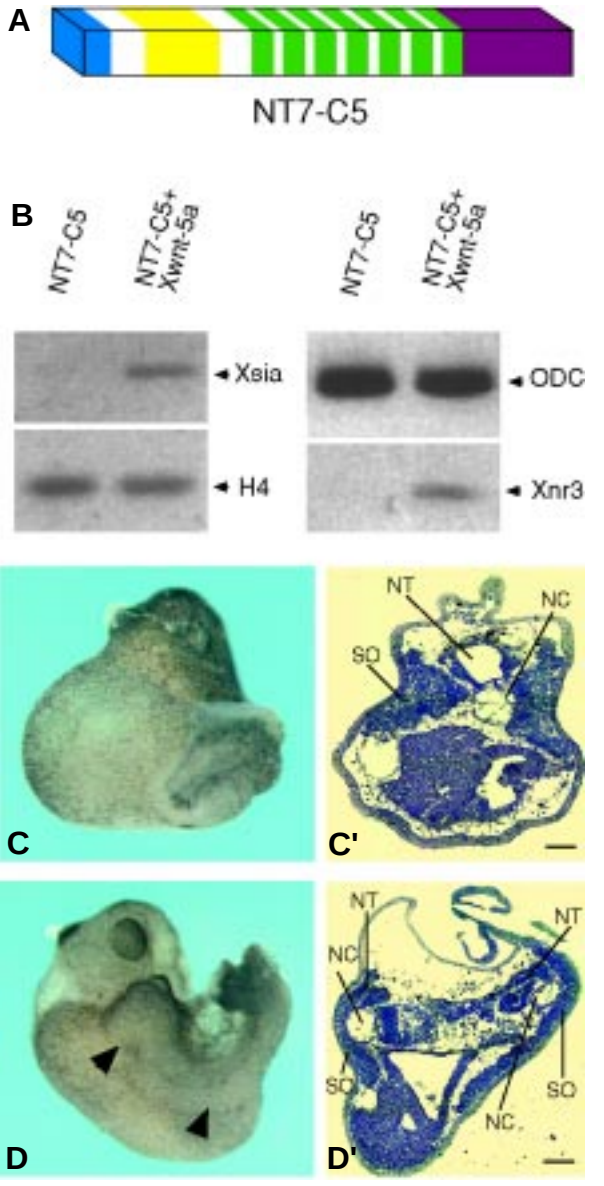

Fig. 3. NT7-C5 expression in animal caps and embryos. **(A)** *Schematic structure of the NT7-C5 construct containing the extracellular and the seven-transmembrane domains of Xfz7 and the carboxy-terminus of Hfz5.* **(B)** *Synthetic mRNA of NT7-C5 (0.5 ng/embryo) and Xwnt-5a (0.1 ng/embryo) was injected into animal blastomeres at the four-cell stage. Animal caps were explanted at stage 8.5 and transcription of Xnr3 and Xsia was analyzed by RT-PCR at stage 10. Xnr3 and Xsia expression was induced only in caps injected with both N5-TC7 and Xwnt-5a mRNA.* **(C)** *Embryos injected with NT7-C5 mRNA into the ventral marginal zone displayed reduced trunk and tail structures (77%, n = 44/58).* **(C′)** *Histological cross section through a NT7-C5-injected specimen.* **(D)** *Embryo expressing NT7-C5 and Xwnt-5a developed a secondary body axis, indicated by arrowheads (85%, n = 53/62).* **(D′)** *Histological cross section through an embryo injected with NT7-C5 and Xwnt-5a mRNA. The secondary axis contains notochord (NC), neural tissue (NT) and somites (SO). Bar represents 100 μm.*

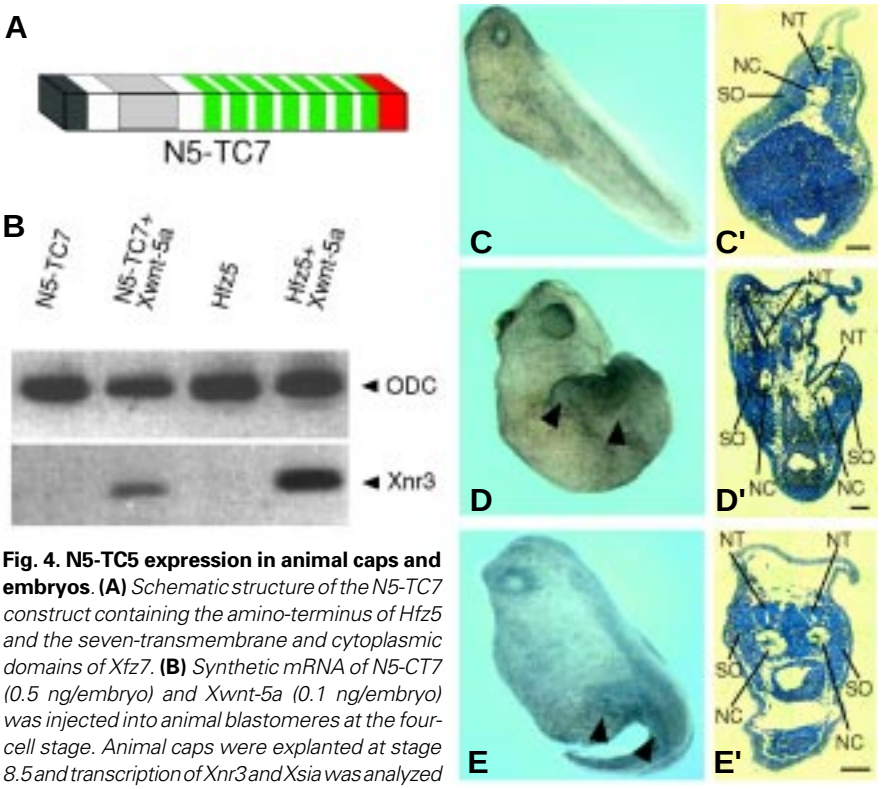

Fig. 4. N5-TC5 expression in animal caps and embryos. **(A)** *Schematic structure of the N5-TC7 construct containing the amino-terminus of Hfz5 and the seven-transmembrane and cytoplasmic domains of Xfz7.* **(B)** *Synthetic mRNA of N5-CT7 (0.5 ng/embryo) and Xwnt-5a (0.1 ng/embryo) was injected into animal blastomeres at the four-cell stage. Animal caps were explanted at stage 8.5 and transcription of Xnr3 and Xsia was analyzed by RT-PCR at stage 10. ODC or H4 were used as RNA controls. Xnr3 and Xsia expression were induced in caps injected with both N5-TC7 or Hfz5 and Xwnt-5a mRNA.* **(C)** *Embryos injected with N5-TC7 mRNA into the ventral marginal zone displayed no morphological defects (96%, n = 49/51).* **(C')** *Histological cross section through a N5-TC7-injected specimen.* **(D)** *Embryos expressing N5-TC7 and Xwnt-5a (62%, n = 33/53) or* **(E)** *Hfz5 and Xwnt-5a (67%, n = 20/30) developed a secondary axis, as indicated by arrowheads. Histological cross section through an embryo injected with* **(D')** *N5-TC5 and Xwnt-5a and* **(E')** *Hfz5 and Xwnt-5a. The secondary axis contains notochord (NC), neural tissue (NT) and somites (SO). Bar represents 100 μm.*

hypothesis, chimeric frizzled receptors were constructed, such that only putative ligand binding domain or seven-transmembrane and cytoplasmic domains involved in signal transduction were exchanged between Xfz7 and Hfz5. *Xfz7* has been shown to activate the Wnt/Ca^{2+} pathway when overexpressed alone and induces the Wnt/β-catenin pathway in combination with *Xwnt-8b* (Medina *et al.*, 2000). *Hfz5*, when overexpressed in combination with *Xwnt-5a*, triggers the Wnt/β-catenin pathway in *Xenopus* but this induction can be inhibited by coexpression of *Xfz7* (Fig. 1). This suggests that Xfz7 can interact with Xwnt-5a but that this receptor ligand combination does not trigger the Wnt/β-catenin pathway.

The Xfz7/Hfz5 chimeras activate the Wnt-1 pathway in a Xwnt-5a-dependent manner

Expression of *NT7-C5* or *N5-TC7* demonstrated that these chimeric receptors do not activate the Wnt/β-catenin pathway in absence of any exogenous ligand (Figs. 3, 4). Expression of *NT7-C5* mRNA, however, resulted in embryos with shortened trunk and tail structures (Fig. 3). This phenotypic effect could be due to the ability of this protein to interfere with zygotic Wnt signaling involved in axis specification at later stages of *Xenopus* development (Itoh and Sokol, 1999). It is possible that this chimeric construct binds to a zygotic Wnt protein necessary for axis specification but cannot transmit the signal. The result of such an interaction would be the loss of function of this Wnt protein. Because NT7-C5 chimeric receptor contains the ligand-binding domain of Xfz7 and therefore, can bind to the same ligand(s) that Xfz7 can bind, it is possible that the underlying mechanism for the effect of this construct on trunk formation is similar to that suggested for Xfz7 (Medina *et al.*, 2000). In contrast, embryos injected with *N5-TC7* developed normally, like their uninjected siblings (Fig. 4), suggesting that this chimeric frizzled receptor containing the ligand binding domain of Hfz5 does not interfere with the zygotic Wnt signaling pathway(s) active at this developmental stage of the embryos. Ectopic expression of *NT7-C5* or *N5-TC7* in combination with *Xwnt-5a* induced the transcription of *Xnr3* and *Xsia* in animal caps and induced secondary axes in embryos (Figs. 3,4). These results show that NT7-C5 can trigger the Wnt/β-catenin pathway in combination with Xwnt-5a in similar manner to Hfz5/Xwnt-5a. Furthermore, it argues that the extracellular domain of Xfz7 can interact with Xwnt-5a. This is also supported by the fact that this domain of Xfz7 can compete with Hfz5 for the Xwnt-5a ligand (Fig. 1). The carboxy-terminal domain of Hfz5 is responsible for activation of the Wnt/β-catenin pathway by this chimeric receptor. The results obtained with *N5-TC7* plus *Xwnt-5a* overexpression confirm that the intracellular part of Xfz7 is able to transmit the Wnt/β-catenin signal (Fig. 4). Despite this ability, Xfz7 cannot activate the Wnt/β-catenin pathway in combination with Xwnt-5a (Medina *et al.*, 2000). This demonstrates that neither the extracellular domain nor the carboxy-terminal cytoplasmic domain of the frizzled receptors are solely responsible for differentially activating the Wnt/β-catenin or the Wnt/Ca^{2+} pathways. A correct combination of the extracellular and the cytoplasmic domains are necessary to selectively activate either of these two pathways.

The transmembrane domains are not required for the transmission of a Wnt/β-catenin signal

It would be interesting to investigate the role played by the seven-transmembrane domains of the frizzled receptors. These transmembrane domains have sequence homology with that of G-protein coupled receptors (Barnes *et al.,* 1998). It has also been shown that some members of frizzled family can modulate the G-protein mediated signaling pathways (Miller *et al.*, 1999). In the case of NT7-C5, the carboxy-terminal domain of Hfz5 reverses the behaviour of Xfz7 in the presence of Xwnt-5a. Though Xfz7 activates the Wnt/Ca^{2+} pathway, it can trigger the Wnt/β-catenin pathway in combination with Xwnt-5a, only when its carboxy-terminus is replaced by that of Hfz5. In this experimental situation, the seven-transmembrane domains do not play a role in Wnt/β-catenin signaling. The ability, however, of these chimeric receptors to activate the Wnt/Ca^{2+} pathway has not yet been tested. It will be interesting to see whether the Xfz7/Hfz5 chimeras can modulate intracellular calcium levels, can activate PKC in combination with

Wnt-5a-type ligands and whether the seven-transmembrane domains play a role in the Wnt/Ca^{2+} pathway.

Materials and Methods

Construction of frizzled receptor chimeras

The extracellular, seven-transmembrane and the cytoplasmic domains of *Xfz7* and *Hfz5* were amplified by PCR and were cloned into a pCS2+ vector. Oligonucleotides used to amplify the receptor fragments were synthesized at MWG-Biotech AG (Ebersberg, Germany). The following forward and reverse primers were used to amplify the extracellular, seven-transmembrane and the cytoplasmic domains of Xfz7 and Hfz5:

Extracellular domain of *Xfz7 (NXfz7)*
NXfz7 F: 5'-GGA ATT CCA GCA TGT CCT CTA CAG TCT CGC TG-3'
NXfz7 R: 5'-TAA CTC GAG AAA GTA CAT CAG CCC GTT GGC TTT GC-3'

Seven-transmembrane and cytoplasmic domains of *Xfz7 (TCXfz7)*
TCXfz7 F: 5'-AGA ATT CCA ACG GGC TGA TGT ACT TTA AGG AG-3'
TCXfz7 R: 5'-TGC TCT AGA TCA CAC CGC AGT CTC CCC TTT G-3'

Extracellular and seven-transmembrane domains of *Xfz7 (NTXfz7)*
NTXfz7 F: 5'-GGA ATT CCA GCA TGT CCT CTA CAG TCT CGC TG-3'
NTXfz7 R: 5'-TAA CTC GAG CCA GAT CCA AAA GCT GGA GGT G-3'

Extracellular domain of *Hfz5 (NHfz5)*
NHfz5 F: 5'-CGG GAT CCG CCA CCA TGG CTC GGC CTG ACC C-3'
NHfz5 R: 5'-CGG AAT TCG TGG CGA ACG TGC GCT CGT CGG C-3'

Cytoplasmic domain of *Hfz5 (CHfz5)*
CHfz5 F: 5'-TAA CTC GAG ACG TCG GGC GTC TGG ATC TGG-3'
CHfz5 R: 5'-TCT AGA CTA CAC GTG CGA CAG GGA CAC CTG C-3'

For the construction of the chimeric receptor NT7-C5, the amplified fragments were joined at an XhoI site generated by PCR and cloned into the EcoRI/XbaI sites of pCS2+. For N5-TC7, the fragments were joined at an EcoRI site and cloned into the BamHI/XbaI sites of pCS2+. The fragments and the restriction enzymes were chosen such that the chimeric receptors retained the amino acid sequence of the original molecules. The construction and translation of these chimeric receptors was simulated on a computer using MacVector™ 4.1 software to verify the continuity of the open reading frame. The chimeric receptors were partially sequenced to verify the intact open reading frame. The chimeric receptor, NT7-C5 contains the extracellular, the seven-transmembrane domains of Xfz7 (aa 1-525) and the cytoplasmic domain of Hfz5 (aa 515-585). The chimeric receptor designated as N5-TC7 contains the extracellular region of Hfz5 (aa 1-236) and the seven-transmembrane as well as the cytoplasmic domains of Xfz7 (aa 215-549).

Egg and embryo manipulations

Eggs were obtained from *Xenopus* females injected with 300-400 units of human chorionic gonadotropin (Sigma, Deisenhofen, Germany) and *in vitro* fertilization was done with macerated testes. The jelly coat was removed using a 2% cysteine solution (pH 8) and the embryos were microinjected in 1X MBS-H [88 mM NaCl, 1 mM KCl, 2.4 mM $NaHCO_3$, 0.82 mM $MgSO_4$, 0.41 mM $CaCl_2$, 0.33 mM $Ca(NO_3)_2$, 10 mM HEPES (pH 7.4), 10 μg/ml streptomycin sulfate and 10 μg/ml penicillin]. To analyze the effect of the chimeric receptors on the normal development (phenotype) of *Xenopus* embryos, synthetic mRNA for these constructs was microinjected into two ventral vegetal blastomeres at the four-cell stage. Either 0.5 ng of mRNA for the chimeric receptors alone or in combination with 0.1 ng of *Xwnt-5a* mRNA was microinjected into the embryos. Embryos were allowed to grow until the tailbud stage in 0.1X MBS-H. The formation of secondary axis was scored at neurula and tailbud stages. Embryonic stages were determined according to Nieuwkoop and Faber (1967).

Capped mRNA synthesis

Capped synthetic sense mRNA was generated using mMessage mMachine SP6 kit (Ambion, Austin, TX, USA). Linearized plasmid DNA from *NT7-C5* (linearized with NotI), *N5-TC7* (linearized with NsiI), *Hfz5* (linearized with HindIII) and *Xwnt-5a* (linearized with XbaI) were used as templates for *in vitro* transcription. Templates used for synthesis of capped mRNA from *Xfz7* and *ΔCXfz7* constructs were linearized with Asp718. The restriction endonucleases were purchased from Roche Diagnostics (Mannheim, Germany).

Animal cap assay

For animal cap assays, four-cell stage embryos were injected with synthetic mRNA for the chimeric receptors alone or in combination with *Xwnt-5a* into the animal blastomeres. Animal cap explants were dissected from the mRNA injected as well as from the control (uninjected) embryos at stage 8.5. The explants were cultured in 0.5X MBS-H until the control embryos reached stage 10. Total RNA was isolated from embryos or animal cap explants using Trizol reagent (Life Technologies, Karlsruhe, Germany) and the expression of *Xnr3* and *Xsia* was detected by RT-PCR.

RT-PCR

RNA (500 ng) was transcribed using AMV-reverse transcriptase (Amersham Pharmacia, Freiburg, Germany) at 50ºC for 30 minutes. PCR amplification was done using Taq Polymerase (Amersham Pharmacia, Freiburg, Germany) as described in Niehrs *et al.* (1994). The following primers were used:

Xsia 5'-CCATGATATTCATCCAACTGTGG-3' and 5'-GTTCTCTTCCTAGATCTGGTAC-3' (317 bp, 34 cycles);
H4 5'-AGGGACAACATCCAGGGCATCACC-3' and 5'-ATCCATGGCGGTAACGGTCTTCCT-3' (188 bp, 23 cyc.);
Xnr3 5'-TGAATCCACTTGTGCAGTTCC-3' and 5'-GACAGTCTGTGTTACATGTCC-3' (233 bp, 29 cycles);
ODC 5'-GTCAATGATGGAGTGTATGGATC-3'and 5'-TCCATTCCGCTCTCCTGAGCAC-3' (385 bp, 25 cycles).

Each cycle consisted of 1 minute denaturation, at 94ºC; 1 minute annealing (the annealing temperature was 55ºC for *Xsia* and *H4* and 65ºC for *Xnr3* and *ODC*) and 1 minute extension at 72ºC. A PTC-200 (MJ-Research, Waltham, MA, USA) cycler was used in all experiments. PCR products were separated on 2% agarose gels in TBE buffer.

Histological analysis of embryos

Embryos were fixed in Romeis (25 ml saturated mercuric chloride, 20 ml trichloroacetic acid, 15 ml 37% formaldehyde) and embedded in Technovit (Kulzer, Wehrsheim, Germany). Sections, 2-5 microns thick were prepared and stained with 0.5% Toluidinblue and 1% Borax. Composite pictures of sections were assembled using AnalySIS software (Soft Imaging System, Münster, Germany).

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