Secreted antagonists of the Wnt signalling pathway

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Journal of Cell Science 116, 2627-2634 © 2003 The Company of Biologists Ltd
doi:10.1242/jcs.00623

Summary

The extracellular antagonists of the Wnt signalling pathway can be divided into two broad classes. Both classes of molecule prevent ligand-receptor interactions, but by different mechanisms: members of the first class, which include the sFRP (secreted Frizzled-related protein) family, WIF (Wnt inhibitory factor)-1 and Cerberus, primarily bind to Wnt proteins; the second class comprises certain members of the Dickkopf (Dkk) family, which bind to one subunit of the Wnt receptor complex. In addition, there are other protein interactions that contribute to Wnt antagonist function. Moreover, certain sFRPs and Dkks do not antagonise Wnt function, which suggests that these families have as-yet-undiscovered functions.

Key words: Wnt, Dickkopf, Frizzled, Cerberus

Introduction

The Wnts are a family of secreted glycoproteins characterised by several conserved cysteine residues. There are 19 human *WNT* genes, several of which encode additional, alternatively spliced isoforms (Miller, 2001). Historically, Wnt proteins have been grouped into two classes – canonical and noncanonical – on the basis of their activity in cell lines or in vivo assays. Canonical Wnts (e.g. Wnt1, Wnt3A and Wnt8) stabilise β-catenin, thereby activating transcription of Tcf/LEF target genes. This results in secondary axis formation in *Xenopus* embryos and morphological transformation of some mammalian cell lines. Noncanonical Wnts (e.g. Wnt4, Wnt5A and Wnt11) activate other signalling pathways, such as the planar-cell-polarity (PCP)-like pathway that guides cell movements during gastrulation (Heisenberg et al., 2000) and the Wnt/Ca^{2+} pathway (discovered in zebrafish and *Xenopus*) (reviewed in Kuhl et al., 2000). Noncanonical Wnts can even antagonise the canonical pathway (Torres et al., 1996; Kuhl et al., 2001; Ishitani et al., 2003). However, several Wnt proteins appear to have both canonical and noncanonical properties – for example, Wnt5A, a noncanonical Wnt, induces secondary axis formation when co-expressed with its receptor, Fz5 (He et al., 1997). Thus, the functional classification of Wnts may depend on the repertoire of Wnt receptors in a particular cell type.

The Wnt receptor complex that activates the canonical pathway contains two components: a member of the frizzled (Fz) family (there are 10 of these seven-transmembrane-span proteins in humans) and either one of two single-span transmembrane proteins, low-density-lipoprotein-receptor-related proteins [LRP-5 and LRP-6 (Pinson et al., 2000; Tamai et al., 2000; Wehrli et al., 2000)] (Fig. 1A). Activation of the noncanonical Wnt pathways is mediated by the Fz family Wnt receptors; it is not clear whether this also requires LRP5/LRP6.

Wnt antagonists can be divided into two functional classes, the sFRP class and the Dickkopf class: members of the sFRP class, which includes the sFRP family, WIF-1 and Cerberus, bind directly to Wnts, thereby altering their ability to bind to the Wnt receptor complex (Fig. 1b); members of the Dickkopf class, which comprises certain Dickkopf family proteins, inhibit Wnt signalling by binding to the LRP5/LRP6 component of the Wnt receptor complex (Fig. 1c). Thus, in theory, those antagonists of the sFRP class will inhibit both canonical and noncanonical pathways, whereas those of the Dickkopf class specifically inhibit the canonical pathway.

Most of our knowledge about the Wnt antagonists comes from developmental studies in *Xenopus* and chick, and there are excellent reviews that cover these aspects in more detail (Niehrs et al., 2001; Yamaguchi, 2001). Here, we focus on what is known about Wnt antagonist function at the molecular level.

Discovery of the sFRP family

sFRPs are antagonists that directly bind to Wnts. They were initially given several names, reflecting their simultaneous discovery by different approaches (Jones and Jomary, 2002). There are presently eight known members of the family. A unifying nomenclature now exists for five of these (sFRP1 to sFRP5) (Table 1), although sFRP3 is still better known as FrzB (for Frizzled motif associated with bone development). On the basis of sequence homology, sFRP1, sFRP2 and sFRP5 form a subgroup, as do sFRP3 and sFRP4, which are quite distantly related to the other sFRPs. Sizzled, Sizzled2 and Crescent form a third subgroup, but these have not been identified in mammals. There are conflicting reports on the ability of Sizzled to inhibit Wnt signalling (Salic et al., 1997; Bradley et al., 2000; Collavin and Kirschner, 2003). Note that, with one exception (Illies et al., 2002), sFRPs (and the other Wnt antagonists) have not been found in invertebrates. Despite this, many of them have been demonstrated to inhibit the activity of the *Drosophila* Wnt homologue Wingless (Wg).

sFRP3/FrzB was first purified as a chondrogenic factor found in cartilage (Hoang et al., 1996). It was discovered at the same time in a screen for *Xenopus* dorsal-specific factors – a screen that also led to the identification of Cerberus (Bouwmeester et al., 1996). FrzB contains a characteristic

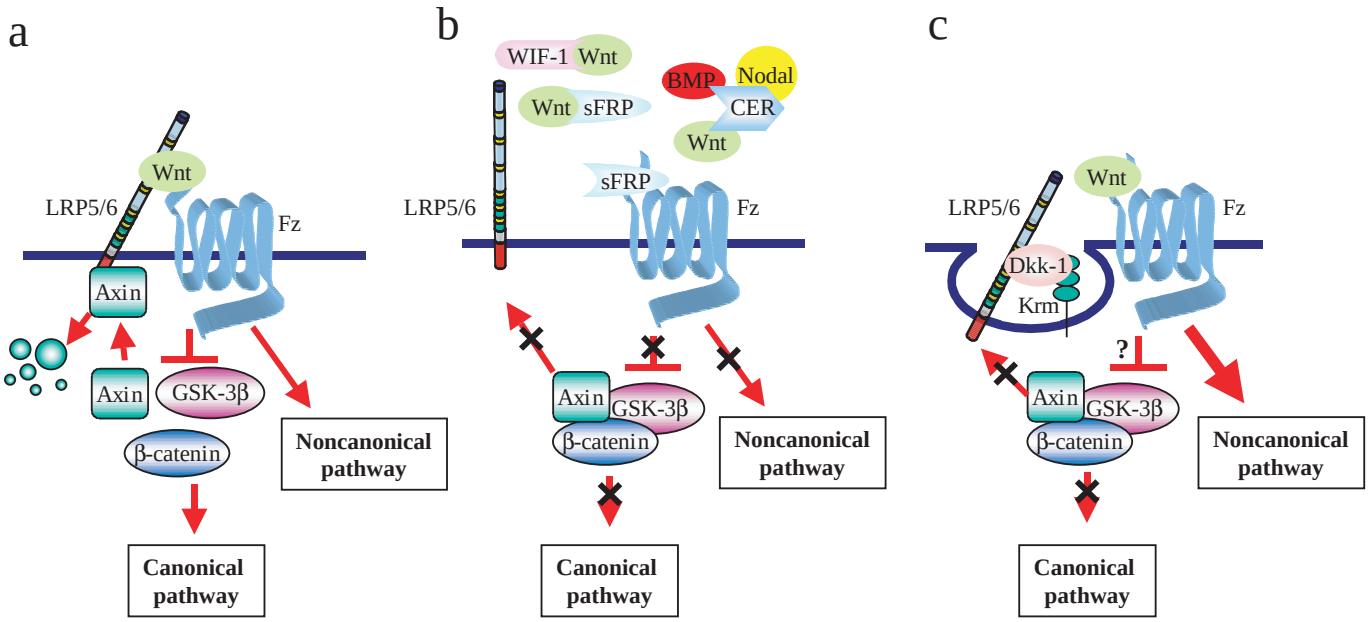

Fig. 1. Regulation of Wnt signalling by antagonists. (a) Activation of the canonical pathway is initiated when Wnt associates with Frizzled (Fz) and LRP5/6. Subsequent events include the recruitment of Axin to LRP5/6 and its degradation, and the phosphorylation of dishevelled, resulting in disruption of the link between β-catenin and GSK-3β. β-catenin is no longer phosphorylated and is thus stabilised. Activation of the noncanonical pathway may involve interaction of Wnt with Fz in the absence of LRP5/6. (b) Antagonists such as sFRPs, Cerberus (CER) and WIF-1 prevent Wnt from binding to its receptors. In this case, both the canonical and the noncanonical pathways are inactivated. sFRPs may also inhibit Wnt by binding to Frizzled. (c) Dkk-1 interacts with LRP5/6 and the co-receptor Kremen 1/2 (Krm, green), and this triggers LRP5/6 endocytosis, thereby preventing formation of the LRP5/6–Wnt–Frizzled complex. Axin brings together the proteins that promote β-catenin phosphorylation, enabling β-catenin degradation and inhibition of the canonical pathway. The Wnt-Fz complex can still activate the noncanonical pathway.

cysteine-rich domain (CRD) that shares homology with the Fz CRD (Fig. 2a), which led to the prediction that it regulates Wnt signalling. This was borne out by experiments done primarily in *Xenopus* embryos: FrzB is present in the Spemann organiser during early gastrulation in a complementary pattern to Xwnt-8 (Leyns et al., 1997; Wang et al., 1997a); it interacts with Xwnt-8 (Wang et al., 1997a) and Wnt-1 (Wang et al., 1997b; Leyns et al., 1997); it inhibits ectopic Xwnt-8 function (Leyns et al., 1997; Wang et al., 1997a); and it inhibits Wnt-1-induced accumulation of β-catenin in cultured cells (Lin et al., 1997).

Members of the sFRP family were also cloned in a search of the EST database for homologs of Fz (Rattner et al., 1997), during the purification of hepatocyte growth factor/scatter factor from the heparin-binding fraction of human embryonic lung fibroblast conditioned medium (Finch et al., 1997), and as proteins secreted by quiescent 10T1/2 fibroblast cells that modulate sensitivity to proapoptotic reagents (Melkonyan et al., 1997). In addition, the ‘secreted frizzled’ Sizzled was found in an expression cloning screen in *Xenopus* embryos (Salic et al., 1997), and Crescent, another sFRP-related molecule, was isolated from chick (Pfeffer et al., 1997). Similarly to FrzB, the inhibitory effects of many of these sFRP family members on Wnt signalling have been demonstrated in *Xenopus* embryos and/or in cultured cells.

Structure/function relationships of sFRPs

The CRDs of sFRPs, which lie in the N-terminal half of the protein (Fig. 2a), share 30-50% sequence similarity with those of Fz proteins and include 10 conserved cysteine residues (Melkonyan et al., 1997). It is remains unclear whether sFRPs antagonise Wnt signalling by interacting with Wnt ligands through the CRD (Lin et al., 1997) or the C-terminal domain, which lies outside the CRD (Uren et al., 2000). The conflicting data may result from differential affinities among sFRPs and their Wnt partners or the use of different ligands (Wg by Uren et al., and Wnt-1 by Lin et al.). Importantly, the CRD of sFRP1 also appears to interact with itself and with Fz (Bafico et al., 1999). Thus, sFRPs may block Wnt signalling either by interacting with Wnt proteins to prevent them from binding to Fz proteins or by forming nonfunctional complexes with Fz.

The C-terminal half of sFRPs contains a domain that shares weak sequence similarity with the axon guidance protein netrin (NTR). This NTR module, which is defined by six cysteine residues and several conserved segments of hydrophobic residues, has also been found in tissue inhibitors of metalloproteases and some complement proteins (Banyai and Patthy, 1999). Although sFRPs are secreted, several reports indicate that sFRPs synthesised by cultured cells are mainly found at the plasma membrane and/or in the extracellular matrix. In common with some Wnts, sFRP1 is released into the culture medium upon addition of heparin (Finch et al., 1997). It is thought that the association of sFRPs with heparan sulfate proteoglycans stabilises sFRP-Wnt complexes (Uren et al., 2000) or determines antagonist localisation.

Table 1. Wnt antagonist family molecules

Name(s)	Mechanism of action	Wnt antagonist activity	Wnt agonist activity/inhibition of antagonist activity
sFRP1 (FRP, SARP2, FrzA) SFRP1	Binds Wnt and Fz	Yes (Finch et al., 1997; Xu et al., 1998; Uren et al., 2000)	Agonist at low concentrations (Uren et al., 2000)
sFRP2 (SARP1) SFRP2	Binds Wnt	Yes (Ladher et al., 2000)	Inhibits sFRP1 (Yoshino et al., 2001)
sFRP3 (FrzB, Fritz) FRZB	Binds Wnt	Yes (Leyns et al., 1997; Wang et al., 1997a; Wang et al., 1997b; Lin et al., 1997; Mayr et al., 1997)	
sFRP4 (FrzB-2) SFRP4	Binds Wnt	?	
sFRP5 (SARP3) SFRP5	Binds Wnt	?	
Sizzled*	Binds Wnt (?)	Yes (Salic et al., 1997) No (Bradley et al., 2000; Collavin and Kirschner, 2003)	
Sizzled2*	Binds Wnt (?)	No (Bradley et al., 2000)	
Crescent*	Binds Wnt	Yes (Pera and De Robertis, 2000)	
WIF-1	Binds Wnt	Yes (Hsieh et al., 1999)	
Cerberus*	Binds Wnt	Yes (Piccolo et al., 1999)	
Coco*	Binds Wnt (?)	Yes (Bell et al., 2003)	
Dkk-1 (DKK1)	Binds LRP5/6	Yes (Glinka et al., 1998; Krupnik et al., 1999; Fedi et al., 1999; Wu et al., 2000; Brott and Sokol, 2002)	Inhibits Dkk-2 (Wu et al., 2000)
Dkk-2 (DKK2)	Binds LRP5/6	No (in *Xenopus* embryos, Krupnik et al., 1999; Wu et al., 2000) Yes (in *Xenopus* embryos, Brott and Sokol, 2002) Yes (in cell lines, Wu et al., 2000; Li et al., 2002)	Agonist (Wu et al., 2000) Agonist with LRP6 (Li et al., 2002; Brott and Sokol, 2002)
Dkk-3 (REIC) (DKK3)		No (Krupnik et al., 1999; Mao and Niehrs, 2003)	
Dkk-4 (DKK4)	Binds LRP5/6 (?)	Yes (Krupnik et al., 1999; Mao and Niehrs, 2003)	
Soggy (DKKL2)		No (Krupnik et al., 1999)	

Approved gene symbols are underlined.
*To date, mammalian genes for Sizzled, Sizzled2 and Crescent have not been identified. The mammalian gene related to Cerberus (mCer1) does not encode a Wnt antagonist, and the antagonist activity of mammalian Coco has not been tested.

sFRP expression patterns during development

The expression patterns of several sFRPs have been examined in developing chick, mouse and *Xenopus* embryos (for details, see Jones and Jomary, 2002). In some cases, the patterns of sFRP expression complement those of specific Wnts, which might support the idea that they antagonise Wnt function. One interesting possibility is that sFRPs control morphogenetic gradients of Wnt signalling activity. Indeed, it has been suggested that sFRPs facilitate boundary definition in the developing organism by limiting the range of Wnt activity. Thus a gradient of sFRP expression might produce gradients of active Wnt protein in regions where a Wnt is expressed uniformly.

Another possibility is that the overlapping patterns of expression of sFRPs simply reflect the regulation of *sFRP* expression by Wnts. *sFRP2* expression in the aggregating mesenchyme, for example, is induced by Wnt-4, which is

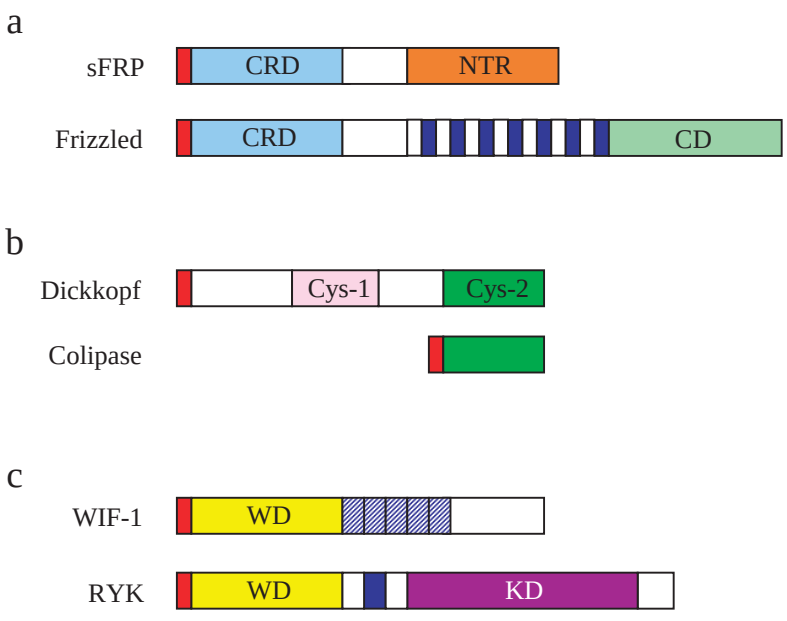

Fig. 2. Wnt antagonists and related proteins. (a) sFRP family proteins are related to Frizzled receptors in the CRD (Cysteine-rich domain). NTR, netrin-like domain; CD, cytoplasmic domain. (b) Dickkopf family proteins are related to pancreatic colipase in Cys-2 (Cysteine-rich domain-2). The domain containing 10 conserved cysteine residues in colipase is shown in green. Cys-1, Cysteine-rich domain-1. (c) WIF-1 is related to the receptor tyrosine kinase, RYK in the WIF domain (WD). KD, tyrosine kinase domain. Signal peptides, transmembrane domains and EGF-like repeats are shown as red, blue, and hashed boxes, respectively.

critical for kidney development at this early stage (Lescher et al., 1998). Interestingly, there is also evidence for opposing gradients of *sFRP1* and *sFRP3* expression in the developing mouse telencephalon (Kim et al., 2001). In order to understand the significance of these expression patterns, we first need to know more precisely to which Wnts each sFRP can bind and whether the sFRP acts as an antagonist or an agonist (see below).

sFRPs can potentiate Wnt activity

When considering sFRP function during development, one should be open to the possibility that sFRPs do not (always) function as Wnt antagonists. Tissue culture experiments have shown that, at low concentrations, sFRP1 potentiates Wg activity rather than inhibiting it (Uren et al., 2000). This and other observations have led to the suggestion that sFRP1 has low-affinity and high-affinity binding sites for Wg; binding to the high-affinity site would promote Wg signalling whereas binding to the low-affinity site would inhibit it. One caveat to these experiments is that they involve Wg rather than a vertebrate Wnt. Although there is a high degree of conservation between Wg and vertebrate Wnts, it is possible that sFRP1-Wg interactions do not represent those between sFRP-1 and Wnts from the same species. An alternative hypothesis is that, at high concentrations, sFRP1 binds to the Fz receptor to form a nonfunctional receptor complex. Indeed, an interaction has been demonstrated in the case of sFRP1 and HFz6 (Bafico et al., 1999). Perhaps the primary role of sFRPs is to transport Wnts to cellular sites that have a high concentration of receptors, where they can be released as active ligands. In this scenario, sFRPs would appear to act as antagonists only at sites where there are few receptors.

The story is further complicated by observations that sFRP1 and sFRP2 elicit different cellular responses when used at similar concentrations. For example, they have opposite effects both on β-catenin stability and cell sensitivity to cytotoxic stimuli in MCF-7 breast cancer cells (Melkonyan et al., 1997). In addition, although both sFRP1 and sFRP2 are expressed in the metanephric kidney, sFRP1 blocks kidney tubule formation and bud branching in cultures of embryonic rat metanephros, whereas sFRP2 has no effect. In fact, sFRP2 blocks the effects of sFRP1 (Yoshino et al., 2001). The major Wnt family member implicated in this process is Wnt-4, and both sFRP1 and sFRP2 are capable of regulating the activity of Wnt-4. The discrepancy may result from differential affinities for Wnt-4 or another Wnt expressed in the kidney. Perhaps, when purified Wnts become available, analysis of the relative affinities of members of the sFRP family for members of the Wnt family will help us to interpret these observations.

sFRPs and the regulation of cell growth

In addition to its roles during development, the Wnt pathway plays important roles in proliferation, differentiation and apoptosis in adult tissues. Thus aberrant activation of the Wnt pathway has been found to occur during tumorigenesis. The frequent downregulation of sFRPs in carcinomas and their upregulation in some degenerative diseases points to their importance in controlling Wnt activity in healthy tissue.

The *sFRP1* gene is found at chromosome 8p21, a site of frequent loss of heterozygosity in human tumours (Wright et al., 1998). It is downregulated in cervical carcinoma (Ko et al., 2002), breast carcinoma (Ugolini et al., 2001) and ovary and kidney carcinomas (Zhou et al., 1998). Moreover, hypermethylation of the *sFRP1* promoter (as well as those of *sFRP2*, *sFRP4* and *sFRP5*) occurs at a high frequency in primary colorectal carcinomas (Suzuki et al., 2002). Tumour cells may shut down the expression of sFRPs because these proteins can promote apoptosis. sFRP1, for example, sensitises MCF-7 breast cancer cells to TNF-induced apoptosis (Melkonyan et al., 1997). sFRPs might also play a pro-apoptotic role in other diseases. sFRP2, for example, is upregulated in the retinas of patients who have retinitis pigmentosa, an apoptotic disease of the retina (Jones et al., 2000).

There are examples in which sFRP expression appears to be incompatible with cell growth in normal tissues. Bovine sFRP1 (called FrzA) is expressed during the formation of neovessels and becomes undetectable when the vasculature is fully mature. It inhibits the growth of endothelial cells (Duplaa et al., 1999), induces angiogenesis in chick chorioallantoic membranes and increases migration and organisation of endothelial cells into capillary-like structures (Dufourcq et al., 2002).

There are also examples in which sFRPs appear to play a positive role in cell growth; sFRP4, for example, is expressed in the stromal cells surrounding endometrial and breast carcinomas but is barely detectable in the stroma of secretory or menstrual endometrium (Abu-Jawdeh et al., 1999). Moreover, in contrast to sFRP1, sFRP2 enables MCF-7 cells to resist TNF-induced apoptosis (Melkonyan et al., 1997). Similar disparities are found in glioma-derived cell lines in which sFRP1 and sFRP2 are upregulated. Although neither sFRP affects glioma cell proliferation nor sensitivity to apoptotic stimuli in vitro, they both confer resistance to serum starvation, and sFRP2 (but not sFRP1) promotes tumour growth in nude mice (Roth et al., 2000). Given the limited number of systems studied, the precise mechanism by which sFRPs regulate cell proliferation and apoptosis remains poorly understood. As we have already discussed, the contradictory effects of sFRPs in some studies might reflect the repertoire of Wnts present, the relative affinities of different sFRPs for Wnts, tissue-specific responses to growth and apoptotic stimuli or biphasic responses to different concentrations of sFRPs.

WIF-1

WIF-1 was first identified as an expressed sequence tag from human retina with highly conserved orthologues in *Xenopus* and zebrafish (Hsieh et al., 1999). The phenotype induced by injection of RNA encoding WIF-1 into early *Xenopus* embryos, namely induction of a partial secondary axis and abnormal somitogenesis, suggested it played a role in the Wnt signalling pathway (Hsieh et al., 1999). Indeed, although WIF-1 does not share any sequence similarity with the CRD domain of Fz or sFRPs, it can bind to XWnt-8 and *Drosophila* Wg in the extracellular space and inhibit Xwnt-8–Dfz2 interactions and Armadillo stabilisation in Xwnt-8-treated *Drosophila* clone-8 cells.

WIF-1 has an N-terminal signal sequence, a unique WIF

domain (WD) that is highly conserved across species, and five epidermal growth factor (EGF)-like repeats highly similar to those of tenascin (Fig. 2c). Interestingly, the WIF domain is also found in the extracellular domain of RYK family (for related to tyrosine kinase) receptor tyrosine kinases, and this has led to the suggestion that RYKs are involved in Wnt signalling (Patthy, 2000). Indeed, the *Drosophila* RYK family member Derailed was recently found to interact both genetically and biochemically with *Drosophila* Wnt5 (but not with *Drosophila* Wnt4 or Wg) to regulate axon guidance (Yoshikawa et al., 2003). However, *Drosophila* Wnt5 is almost twice as large as other Wnt family members, and it is not clear whether Derailed binds to the Wnt domain of *Drosophila* Wnt5 or to the unique N-terminal domain.

Cerberus

Cerberus is another Wnt antagonist that belongs to the same class as WIF-1 and sFRPs. It was isolated from *Xenopus* as an abundant organiser-specific molecule (Bouwmeester et al., 1996). It is expressed in the anterior endoderm, including the Spemann's organiser, and has the unique property of inducing an ectopic head without trunk formation. Trunk formation relies on Nodal and Wnt signalling, whereas head induction requires inhibition of Wnt and bone morphogenetic protein (BMP) signalling. Cerberus, as a multivalent growth-factor antagonist, inhibits all three signalling pathways, which leads to simultaneous head formation and trunk inhibition. It has a cysteine-knot domain that is found in several cytokines, including members of the transforming growth factor-β (TGF-β) superfamily and their antagonists (Biben et al., 1998; Piccolo et al., 1999). Proteolytically processed isoforms of *Xenopus* Cerberus (XCer), which still contain the cysteine-knot, can bind to nodal-related-1 but not to Wnt-8 and BMP-4; so the ability of XCer to act as a Wnt antagonist might be regulated by proteolysis (Piccolo et al., 1999). XCer is distantly related to the mouse protein, Cerberus-like (mCer1), but mCer1 has not been shown to affect Wnt signalling, and it is unclear whether it is a true mouse orthologue (Belo et al., 2000). Recently, another *Xenopus* Cerberus-like molecule was discovered, named Coco, which can also inhibit Wnt signalling (Bell et al., 2003).

The Dickkopf family

The Dkk family comprises four members (Dkk-1 to Dkk-4) and a unique Dkk-3-related protein named Soggy (Sgy). Dkks contain two characteristic cysteine-rich domains (Cys-1 and Cys-2) separated by a linker region of variable length (Glinka et al., 1998; Krupnik et al., 1999) (Fig. 2b). Cys-2, in particular, is highly conserved among all members of the family and contains 10 conserved cysteine residues; this is similar to the proteins of the colipase family, with which Dkk proteins share weak sequence similarity (Aravind and Koonin, 1998; Krupnik et al., 1999). Detailed protein sequence and structural analysis suggested that Dkks and colipase have the same disulfide-bonding pattern and a similar fold. Colipases are essential for lipid hydrolysis by pancreatic lipases and interact with lipid micelles (van Tilbeurgh et al., 1999). It is not known whether the structural similarity between colipases and Dkks implies a common function such as lipid interaction.

Dkks and development

The most studied member of the Dkk family is Dkk-1. The characteristic developmental function of Dkk-1 is its head-inducing activity. Dkk-1 was originally cloned as a molecule that is able to induce secondary axes with a complete head when its mRNA is injected into *Xenopus* embryos together with a dominant-negative mutant of the BMP-2/4 receptor (Glinka et al., 1998). Dkk-1 is expressed in the anterior endomesoderm of the Spemann organiser, where head-inducing activity exists, and injection of Dkk-1 mRNA results in anteriorisation with an 'enlarged head' phenotype. Moreover, *Xenopus* embryos injected with an anti-Dkk-1 antibody (Glinka et al., 1998) and Dkk-1-knockout mice (Mukhopadhyay et al., 2001) lack anterior head structures, indicating that Dkk-1 is essential for head formation.

Similarly to sFRP3/FrzB, Dkk-1 blocks both the early and late effects of ectopic Xwnt-8 in *Xenopus* embryos (Glinka et al., 1998) and inhibits Wnt-induced stabilisation of β-catenin (Fedi et al., 1999) and β-catenin/Tcf-dependent transcription of both artificial and endogenous genes in mammalian and amphibian cells, respectively (Wu et al., 2000; Brott and Sokol, 2002). However, unlike sFRPs, Dkk-1 prevents activation of the Wnt signalling pathway by binding to LRP5/6 rather than to Wnt proteins (Bafico et al., 2001; Mao, B. et al., 2001; Semenov et al., 2001).

In addition to LRP5/6, Dkk-1 interacts with another class of receptor, the single-pass transmembrane proteins Kremen1 (Krm1) and Kremen2 (Krm2) (Mao et al., 2002). Krm, Dkk-1 and LRP6 form a ternary complex that disrupts Wnt/LRP6 signalling by promoting endocytosis and removal of the Wnt receptor from the plasma membrane (Mao et al., 2002). Studies in *Xenopus* indicate that Krm proteins inhibit Wnt activity during early anteroposterior patterning of the central nervous system: overexpression of Krm anteriorises embryos and rescues embryos posteriorised by Wnt8, and antisense knockdown of Krm1 and Krm2 leads to deficiency of anterior neural development (Davidson et al., 2002). Although a precise molecular mechanism describing how the Krm–Dkk-1–LRP6 complex inhibits the canonical Wnt signalling pathway remains unclear, there are some clues. A key component of the canonical Wnt pathway is Axin, which negatively regulates Wnt signalling by facilitating the phosphorylation of β-catenin, marking it for proteosomal degradation. Wnt-activated LRP-5 recruits Axin to the plasma membrane and promotes its degradation, thereby leading to the stabilisation of β-catenin (Mao, J. et al., 2001). By promoting the internalisation of LRP5/6 through Krm, Dkk-1 might inhibit recruitment of Axin to the plasma membrane.

Activation of the noncanonical Wnt PCP-like pathway, which triggers convergent extension movements during gastrulation, is inhibited by dominant-negative Fz, but not by Dkk-1 or by dominant-negative LRP6 (Semenov et al., 2001). Thus, the antagonistic effect of Dkk-1, mediated by LRP5/6, is likely to be specific to the Wnt/β-catenin pathway. However, is too early to draw a definitive conclusion since it was recently shown that Dkk-1 could activate the noncanonical PCP-like pathway (Pandur et al., 2002) (although GSK-3β, which also inhibits the canonical pathway, had a similar effect in these experiments).

To date, the Wnt antagonist activity of Dkk-4 appears to be indistinguishable from Dkk-1, whereas Dkk-3 and Sgy have no

effect on Wnt signalling (Krupnik et al., 1999; Mao and Niehrs, 2003). However, Dkk-2 is more complicated. Although both Dkk-1 and Dkk-2 can bind to LRP6 and Krm2 (Mao et al., 2002) and antagonise β-catenin/Tcf-dependent transcription induced by Wnt-1 and Xwnt-8 (Wu et al., 2000; Brott and Sokol, 2002), Dkk-2 is a poor inhibitor of Xwnt-8-induced axis duplication (Krupnik et al., 1999; Wu et al., 2000). [This may, in part, be because Dkk-2 cannot be expressed to such high levels as Dkk-1 (Brott and Sokol, 2002).] Moreover, ectopic expression of Dkk-2 (but not Dkk-1) activates Wnt/β-catenin signalling in *Xenopus* embryos (Wu et al., 2000), and Dkk-2 (but not Dkk-1) synergises with LRP6 to promote axis duplication and activation of the *Siamois* promotor (Brott and Sokol, 2002).

Analysis of deletion mutants and chimeric proteins indicates that the C-terminal domains of Dkk-1 and Dkk-2, which contain the Cys-2 region, behave similarly to one another: in isolation they are necessary and sufficient for association with LRP6, potentiation of LRP6-induced axis induction, and transcriptional activation of reporter genes (Brott and Sokol, 2002; Li et al., 2002; Mao and Niehrs, 2003), and they inhibit Xwnt-8-dependent secondary axis formation and cooperate with a dominant-negative BMP-4 receptor to promote head induction (Brott and Sokol, 2002). This suggests that the different activities of Dkk-1 and Dkk-2 might result from differences in their N-terminal domains. Indeed, when the N-terminal domain of Dkk-1 is fused to the C-terminal domain of Dkk-2, it inhibits the ability of the latter to synergise with LRP6 to activate Wnt signalling (Brott and Sokol, 2002). One possibility is that the N-terminal domain of Dkk-1 prevents LRP6-Fz interactions. The Cys-2 region also contains the binding site for Krm1/2, and the co-expression of Krm2 is sufficient to convert Dkk-2 from an LRP6 agonist into an LRP6 antagonist (Mao and Niehrs, 2003). This suggests that the relative levels of expression of LRP5/6 and Krm1/2 proteins might determine the ability of Dkk-2 to act as an agonist or an antagonist.

Potential cellular functions of Dkks

The expression of Dkk-1 overlaps with sites of programmed cell death during limb development (Mukhopadhyay et al., 2001; Grotewald and Ruther, 2002), and the loss of Dkk-1 expression results in the fusion of digits and formation of ectopic digits similar to those found in mice possessing mutations in other proteins that regulate programmed cell death in the limb (Mukhopadhyay et al., 2001). It remains to be seen whether Dkk-1 inhibition of the Wnt/β-catenin pathway is required for induction of apoptosis. Dkk-1 may be an important mediator of apoptosis induced by a variety of stimuli. The *Dkk-1* promoter contains a p53-responsive element and the expression of Dkk-1 is induced by p53 (Wang et al., 2000). Moreover, genotoxic stress caused by UV and chemotherapeutic agents enhances Dkk-1 expression, and Dkk-1 sensitises glioma cells to ceramide-induced apoptosis (Shou et al., 2002). The increase in Dkk-1 expression induced by several apoptotic agents appears to involve the transcription factor Jun (Grotewald and Ruther, 2002).

It is still unclear whether aberrant expression of Dkk-1 is a causative agent in human disease. However, studies of an inherited *LRP5* mutation indicate a potential role for Dkk-1 in pathogenesis. A congenital Gly171Val mutation occurs in all affected members of a kindred with an autosomal dominant syndrome characterised by high bone density (Boyden et al., 2002; Little et al., 2002). This mutation is refractory to Dkk-1 antagonism and thus may augment the activity of the Wnt pathway (Boyden et al., 2002). This suggests that other Wnt antagonists cannot compensate for this function of Dkk-1/LRP. In mice, disruption *LRP5* leads to a decrease in osteoblast proliferation, which results in a low bone mass phenotype (Kato et al., 2002).

The biological roles of Dkk-3 and Sgy in the Wnt pathway remain unclear because they do not inhibit canonical Wnt signalling (Krupnik et al., 1999; Mao, B. et al., 2001), and Dkk-3 (Sgy has not been tested) does not interact with LRPs or Krm1/2 (Mao, B. et al., 2001; Mao et al., 2002). *Dkk-3* was independently cloned as a gene that has reduced expression in immortalised cells and tumour cell lines (Tsuji et al., 2000). It is frequently downregulated in non-small cell lung cancer and has growth inhibitory effects on tumor cells (Tsuji et al., 2001). It remains to be seen whether Dkk-3 antagonises other growth factor pathways by mechanisms that involve direct association with ligands or transmembrane receptors in a manner similar to that in which Dkk-1 inhibits Wnt signalling. Sgy is related in sequence to Dkk-3 (22% residue identity in humans), in particular within the N-terminal domain, but does not share any homology with other Dkks (Krupnik et al., 1999) and so is not expected to function as a Wnt antagonist. Sgy is expressed specifically in developing spermatocytes, which indicates that it might have a role in spermatogenesis (Kaneko et DePamphilis, 2000).

Conclusion/perspectives

The two major classes of Wnt antagonists function in quite different ways: the sFRP class bind to Wnt ligands; and the Dkk class bind to a component of the Wnt receptor. The outcome appears to be that sFRPs inhibit canonical and noncanonical pathways whereas Dkks inhibit only the canonical pathway. However, recent studies have revealed that neither class of antagonist is quite so simple. Both sFRPs and Dkks can activate the canonical pathway; in some cases one antagonist can inhibit the action of another. Indeed, it may turn out that certain antagonists act as such only when expressed at nonphysiological levels. Clearly there are many unresolved issues. The keys to a better understanding of these proteins will be the outcome of loss-of-function studies and the availability of purified Wnt proteins (Willert et al., 2003) to help determine the binding affinities and specificities of each antagonist.

The authors are supported by the Prostate Cancer Charity.

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