Wnts and Hedgehogs: lipid-modified proteins and similarities in signaling mechanisms at the cell surface

Roel Nusse

Howard Hughes Medical Institute, Department of Developmental Biology, Beckman Center, Stanford University, Stanford, CA 94305, USA
(e-mail: rnusse@cmgm.stanford.edu)

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Summary

This review compares the signaling mechanisms of the Wnt and the Hedgehog proteins. Although Wnts and Hedgehogs are unrelated proteins, they are both modified by lipids, possibly through the action of enzymes that are related to each other. At the surface of target cells, the reception of Wnt and Hedgehog signals is regulated by several molecules, some of which, in particular the Frizzled and Smoothened receptors, are related to each other. Several other aspects of Wnt and Hedgehog transport and signaling are discussed, as well as the possible origin of these pathways.

Introduction

Animal development leads to numerous different outcomes, but the large majority of signaling events in embryos involve only a handful of families of molecules, including the Notch-Delta membrane molecules, the Fibroblast Growth Factors (Fgfs), the Bone Morphogenetic Proteins (Bmps), the Hedgehogs (Hh) and the Wnts. Surprisingly, evidence is emerging that indicates that Wnt and Hh signaling are similar to each other in several respects, inviting speculation that these signaling pathways are evolutionarily related. Among the features they share are lipid-modified signals and the participation of cell-surface receptors Frizzled (Fz) and Smoothened (Smo) that are related to each other. In addition, both Wnt and Hh signaling use the protein kinases Gsk3 and Ck1α to facilitate proteolysis of the key transcriptional effectors: β-catenin for Wnt and Cubitus interruptus (Ci) for Hh (Kalderon, 2002).

This review summarizes our current understanding of Wnt and Hh signaling, focusing on the nature of the proteins, and the interactions between the signals and cell surface molecules in these pathways. The lipid modification of Wnt and Hh poses a number of interesting questions that are central to our understanding of the developmental roles of these molecules. The biochemical aspects of signaling will therefore be discussed in the context of the developmental functions and the genetics of Wnt and Hh signaling. For overviews of Wnt and Hh pathways within cells, an aspect that will not be presented here, the reader is referred to various other reviews (Bienz and Clevers, 2000; Cadigan and Nusse, 1997; Ingham, 2001; Ingham and McMahon, 2001; Kalderon, 2000; Kalderon, 2002; McMahon, 2000; Moon et al., 2002) and the Wnt homepage (see http://www.stanford.edu/~rnusse/wntwindow.html).

Wnt and Hh proteins: lipid-modified signals

Both the Wnt and the Hh proteins, which are not related in sequence to each other, are destined for secretion. Because molecules that are secreted from cells are commonly glycosylated but not, as far as known, acylated, the discovery that Wnt and Hh proteins carry covalently attached palmitates (Pepinsky et al., 1998; Willert et al., 2003) came as a surprise (Fig. 1). In the case of Hh, palmitoylation is one of two lipid modifications, the other one being cholesterol (Porter et al., 1996b).

The Hh protein is made as a precursor molecule, consisting of a C-terminal protease domain and an N-terminal signaling unit, and undergoes a number of unusual modifications during its synthesis. The N terminus of Hh becomes modified by the fatty acid palmitate, on a conserved cysteine residue that is exposed at the very N-terminal end of the protein after its signal sequence has been removed (Pepinsky et al., 1998) (Figs 1, 2; see also Fig. 4). The palmitoyl group is attached through an amide to the NH_2 group of the cysteine, but it is thought that the initial link between palmitate and the cysteine is by a thioesther to the sulfhydryl group, after which the palmitate is transferred to the NH_2 terminus (Pepinsky et al., 1998).

The processing of Hh is unusual in a number of aspects: Hh is made initially as a precursor molecule that consists of a C-terminal protease domain and an N-terminal signaling unit. The C-terminal protease of Hh cleaves the precursor in an autocatalytic manner to release the active signaling domain of Hh called HhNp (Lee et al., 1994). During this cleavage, the C terminus of HhNp becomes covalently modified by a cholesterol molecule (Porter et al., 1996a; Porter et al., 1996b) (Fig. 1). Various forms of Hh have been engineered that lack the autocatalytic cleavage site and are not therefore cholesterol-modified. These variants are called HhN; the mammalian forms of HhN appear not only to lack cholesterol but are also much less efficiently palmitoylated (Pepinsky et al., 1998). Assuming that the relationship between cholesterol modification and palmitoylation is general and also pertains to *Drosophila*, this should be considered in interpreting the relative contributions of the cholesterol and palmitate modifications to Hh activity both in vivo or in different genetic backgrounds (see below).

Wnt genes and Wnt signaling events have been known for

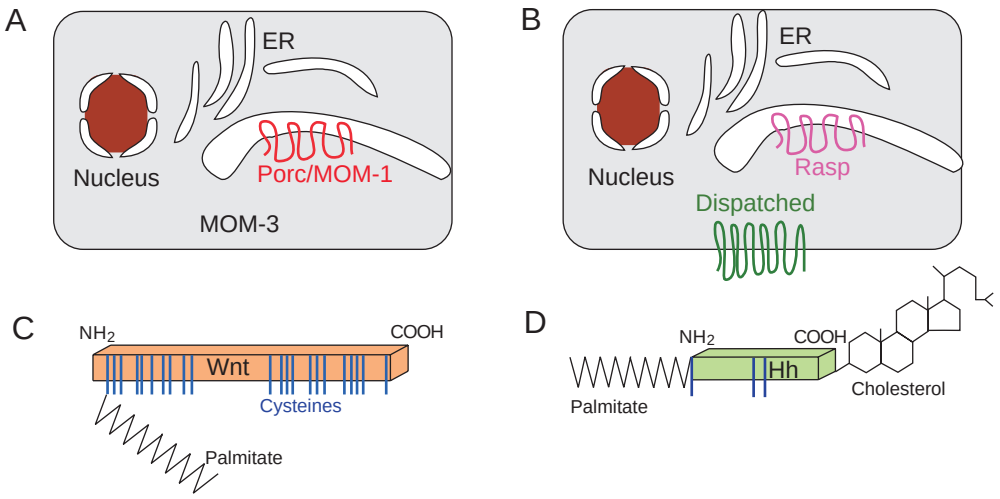

Fig. 1. Production of lipid-modified Wnt and Hedgehog (Hh) proteins. (A) Porcupine (Porc), or MOM-1 in *C. elegans*, is an endoplasmic reticulum (ER) protein that is required in Wnt producing cells, and which is thought to attach a palmitate to Wnt. In *C. elegans*, the MOM-3 gene product (not yet identified molecularly) may assist in the production or release of active Wnt. (B) In Hh-producing cells, Rasp is necessary for the acylation of the Hh protein. The C terminus of Hh is cholesterol-modified. Release of lipid-modified Hh requires the transmembrane protein Dispatched (Disp). (C,D) The Wnt (C) and Hh (D) proteins, showing the approximate positions of cysteine residues (blue vertical lines) and lipid modifications.

several decades (reviewed by Cadigan and Nusse, 1997) but the nature of the active Wnt proteins themselves remained unknown until recently. Persistent problems in solubilizing and isolating active Wnt molecules hindered the biochemical characterization of these proteins, and presented major problems to understanding how Wnt proteins signal. The recent purification of Wnt proteins (Willert et al., 2003) has, to some extent, now explained how Wnts might function by revealing that Wnt molecules are palmitoylated and are therefore much more hydrophobic than was previously predicted from their primary amino acid sequences (Willert et al., 2003) (Fig. 1). The amino acid of Wnt proteins that appears to be modified is the first conserved cysteine (C77), a residue that is present in all Wnts and that is essential for Wnt function, as revealed by mutant analysis (Willert et al., 2003). Treating Wnt with the enzyme acyl protein thioesterase results in a form that is neither hydrophobic nor active, strengthening the evidence that the palmitate is important for signaling. Wnt proteins are, unlike Hedgehog proteins, usually glycosylated on conserved N-linked glycosylation sites (Mason et al., 1992), although it is possible that they carry other modifications as well, or that different forms of Wnt are palmitoylated at different sites.

Wnts and Hedgehogs: similar enzymes for acylation?

Genetic observations had previously indicated that Wnts are lipid modified, as genes with homology to acyltransferases are required for Wnt signaling. These genes, *porcupine* (*porc*) in *Drosophila* (Kadowaki et al., 1996) and *mom-1* in *C. elegans* (Rocheleau et al., 1997), are required in Wnt-producing cells rather than for reception of the signal (Fig. 1). In 2000, Hofmann (Hofmann, 2000) reported that sequence similarities exist between Porc and membrane-bound acyltransferases, enzymes that are present in the Endoplasmic Reticulum (ER) membrane and that acylate a variety of substrates. Tanaka et al. have also observed that Porc can bind to a domain in Wingless (Wg; the best characterized Wnt in *Drosophila*) that encompasses the acylation site, providing more evidence that Porc is the enzyme that is responsible for the acylation of Wnt proteins, although this remains to be shown directly (Tanaka et al., 2002; Willert et al., 2003).

Strikingly, a gene with homology to *porc* is genetically required for the production of active Hh. This gene was isolated by four different groups and is therefore known by the names *skinny hedgehog* (Chamoun et al., 2001), *sightless* (Lee and Treisman, 2001) *central missing* (Amanai and Jiang, 2001) and *rasp* (Micchelli et al., 2002). (I use the FlyBase nomenclature of *rasp* for this gene.) Similar to *porc*, mosaic analysis indicates that *rasp* is required in Hh-producing cells (Micchelli et al., 2002). Cultured cells in which *rasp* function is blocked by RNA interference secrete a form of Hh that lacks palmitate (Chamoun et al., 2001). It is likely, therefore, that Rasp is indeed the enzyme that acylates Hh, but direct biochemical evidence for this is lacking. It is not known where Rasp resides in cells, but, by analogy to Porc, a likely location is the ER (Kadowaki et al., 1996).

A consensus sequence for acylation is unknown, not only for Porc and Rasp, but also for the numerous cytoplasmic proteins that are lipid modified (Dunphy and Linder, 1998). It is nevertheless interesting to note that the cysteines that are lipid modified in Wnt and Hh are followed by several basic residues in either protein (Fig. 2). This indicates that the enzymes encoded by *porc* and *rasp* use a positively charged stretch of amino acids, which is conserved in the known Wnt and Hh proteins (not shown), as a recognition site.

Function of the lipids

The phenotypic similarities between animals with mutations in *wnt* and *porc/mom-1* indicate that Porc and MOM-1 are enzymes that are specific to Wnt signaling, and underscores the significance of the lipid as an integral component of Wnt

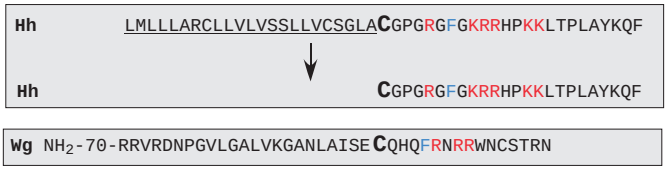

Fig. 2. Positions of palmitoylated cysteines in Hh and Wnt. Palmitoylated cysteines (C) are shown in bold. In Hh, this cysteine is at the N terminus after the signal sequence (underlined) has been removed (Pepinsky et al., 1998). The cysteine in the Wnt protein Wnt3A and, by homology, in Wingless (Wg; shown here), is internal. However, this does not exclude the possibility that Wnt carries additional modifications at other sites. In both Hh and Wnt, there are basic residues downstream of the modified cysteine (shown in red).

activity. In particular, none of the numerous functions of *wg* in *Drosophila* are detectable in the absence of *porc* (Kadowaki et al., 1996), whereas absence of *rasp* does not have any detectable consequences for *wg* (Micchelli et al., 2002).

Although these genetic data indicate that lipid modification is required for Wnt and Hh protein activity, certain conditions can be created under which the palmitate is not absolutely essential for their function. Overexpressing Wg in the fly, under the control of a strong promoter, can partially circumvent the need for Porc function (Noordermeer et al., 1995). Similarly, *wnt* mutant-gene constructs that lack the palmitoylation site can, when overexpressed in cells, produce a weak signal (Willert et al., 2003). In addition, non-palmitoylated Hh protein is active at high concentrations (Pepinsky et al., 1998). It is possible, therefore, that the lipid serves to localize the proteins to membranes and that its absence can be overcome by high protein concentrations. Furthermore, the cysteine that is acylated on the Hh molecule can be substituted with various hydrophobic amino acids (Pepinsky et al., 2000) with some retention of activity, an observation that indicates that the lipid might target Hh to the surface membrane of a cell, where Hh functions.

A site-directed mutation in one of the endogenous Hh genes in the mouse (*sonic hedgehog*) showed that loss of cholesterol modification attenuates the range of Hh activity or perhaps signaling activity itself (Lewis et al., 2001). It was shown earlier that overexpression of cholesterol-free Hh (HhN) can overcome this defect (Beachy et al., 1997). Both observations could imply that cholesterol enhances activity by membrane targeting, but it should be taken into account that these Hh mutants may lack palmitate as well. Indeed, removing *rasp* in *Drosophila* eliminates the effects of overexpressed cholesterol-free HhN (Chamoun et al., 2001). If Rasp is the enzyme that palmitoylates Hh, this interesting observation suggests that the HhN molecules, while free of cholesterol, are to some extent still palmitoylated. This palmitoylated form is then the active species, blocked in its activity by the absence of Rasp.

Transport and release of Wnt and Hh

Lipid attachment is a common modification of cytoplasmic proteins (Dunphy and Linder, 1998) and is important for the membrane targeting of intracellular signaling molecules, but, as far as it is known, it is rare in proteins that operate outside cells. Are Wnt and Hh molecules actually released from cells and, if so, how? Or do they always tether to membranes? It has also been reported that Wnt and Hh proteins can act on cells away from their source, as concentration-dependent, long-range morphogenetic signals (Roelink et al., 1995; Zecca et al., 1996; Zeng et al., 2001). What role, if any, do lipids play in the long-range transport of these proteins?

The release of Hh from cells requires a dedicated transport molecule: a protein called Dispatched (Disp). Initially found in *Drosophila* (Burke et al., 1999) but functionally conserved in mammals (Caspary et al., 2002; Kawakami et al., 2002), Disp is a multiple-pass, transmembrane protein (Fig. 1). In the absence of Disp, Hh is not secreted from cells and is unable to signal to neighboring cells. Interestingly, non-cholesterol modified HhN is not dependent on Disp; it is secreted and is fully active, suggesting that the primary function of Disp is to transport cholesterol-modified Hh (Burke et al., 1999). However, as mentioned above, it should be kept in mind that HhN also lacks palmitate (Pepinsky et al., 1998); it therefore remains possible that Disp is specifically needed for the release of palmitoylated Hh from cells.

There is no evidence of a similar transporter for Wnt molecules, although a gene identified in *C. elegans*, *mom-3*, is required in Wnt-producing cells (Rocheleau et al., 1997). This gene (also called *mig-14*) remains to be characterized molecularly.

It is not clear how the palmitate influences Wnt and Hh transport from one cell to another. Variants of Hh that lack the palmitoylation site are secreted from cells, perhaps more efficiently than is wild-type Hh (Chamoun et al., 2001; Pepinsky et al., 1998). The same is true for Hh protein in the absence of *rasp* (Chamoun et al., 2001); however, the non-palmitoylated Hh protein is not functional.

With respect to secretion, the effect of disrupting palmitoylation of the Wnt proteins is strikingly different to disrupting palmitoylation of Hh. A *wg* allele (S21), in which the palmitoylated cysteine is mutated into a tyrosine (Couso and Arias, 1994; Willert et al., 2003), results in a protein that is not secreted (Fig. 3). The lack of secretion of Wnt-mutant proteins is commonly seen and is usually attributed to protein misfolding. More surprising is the observation that, in the absence of *porc*, Wg is also retained by cells (van den Heuvel et al., 1993) (Fig. 3). At first glance, it might seem paradoxical that a Wnt without lipid is not secreted, in particular in comparison with Hh, as one might expect that a less hydrophobic variant is better released from cells. This difference is likely explained by the overall structure of Wnt, which is a molecule that is rich in cysteines that are presumably disulphide linked. In the absence of *porc*, the C77 residue will have a free sulfhydryl group that may interfere with normal disulphide formation of other cysteines, leading to a misfolded and retained protein (Fig. 4). The palmitate on Hh is also attached to a cysteine, but through an amide at the N terminus, which leaves the sulfhydryl group free. Thus, the lack of palmitate on Hh (in the *rasp* mutant) does not change the overall number of free sulfhydryl groups (Fig. 4). In fact, none of the three cysteines in Hh are involved in disulphide formation (Hall et al., 1997).

Irrespective of these differences, the intriguing question of how lipid-modified Wnt and Hh molecules are transported remains. Are there are carrier molecules that bind directly to the lipids? Or are the proteins always linked to membranes, even when shuttled between cells? Evidence has been

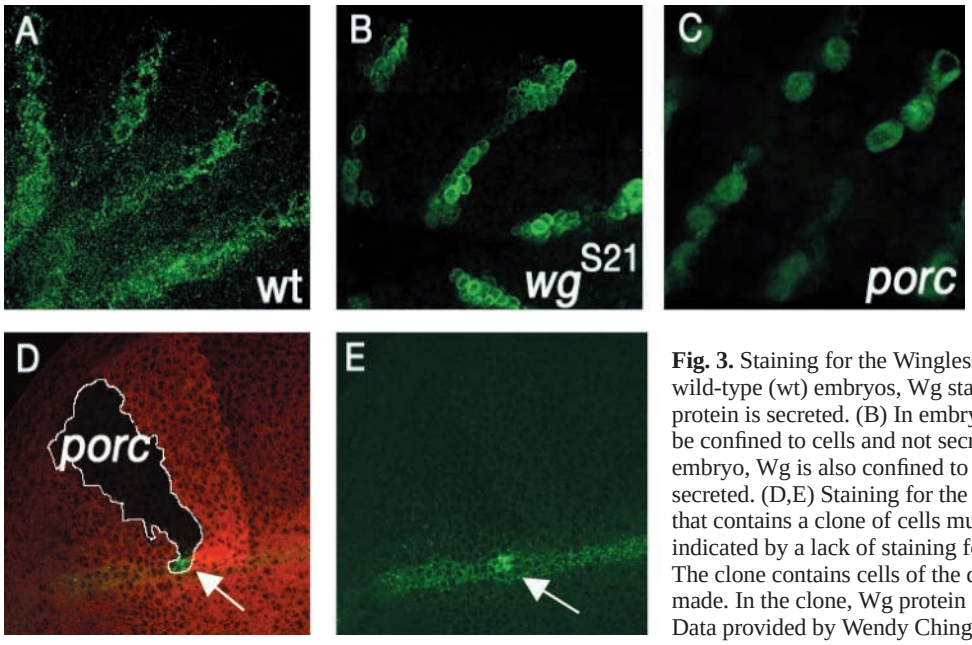

Fig. 3. Staining for the Wingless (Wg) protein in *Drosophila* tissues. (A) In wild-type (wt) embryos, Wg staining (green) is diffuse, indicating that the protein is secreted. (B) In embryos carrying the wg^{S21} allele, Wg appears to be confined to cells and not secreted. (C) In a *porcupine* (*porc*)-mutant embryo, Wg is also confined to producing cells, indicating that it is not secreted. (D,E) Staining for the Wg protein (green) in a wing imaginal disc that contains a clone of cells mutant for *porc*. The position of the clone is indicated by a lack of staining for β-galactosidase (red) and is outlined (D). The clone contains cells of the dorsal-ventral wing boundary where Wg is made. In the clone, Wg protein accumulates and is not secreted (arrows). Data provided by Wendy Ching (see also van den Heuvel et al., 1993).

presented for a freely diffusible form of Shh that is cholesterol modified and multimeric (Zeng et al., 2001). In vivo, vesicle-based transport outside of cells has been proposed to exist in *Drosophila* wing imaginal discs; the vesicles have been called argosomes and might carry Wg protein (Greco et al., 2001). Other structures that may play a role in transporting Wnts are cytonemes, long cellular processes that perhaps carry ligands and receptors away from cells (Ramirez-Weber and Kornberg, 1999). Much work in this area remains to be done, but as answering these questions is central to our understanding of developmental mechanisms in general, there will no doubt be significant interest in further experimental tests.

Proteoglycans in Wnt and Hh transport?

Heparin-sulfated forms of proteoglycans (HSPG) are long proteins with branched sugar side chains that are expressed on the cell surface, often through a GPI anchor (Nybakken and Perrimon, 2002). HSPGs can form complexes with a variety of signaling molecules, including Fgfs and Wnts (Nybakken and Perrimon, 2002). Genetic evidence has implicated HSPGs in Wnt signaling, both in *Drosophila* (Lin and Perrimon, 1999; Tsuda et al., 1999), and in mouse mammary tumorigenesis (Alexander et al., 2000). Absence of *Dally*, a gene encoding an HSPG in *Drosophila*, generates phenotypes similar to *wg* (Lin and Perrimon, 1999; Tsuda et al., 1999), as do mutations in genes that encode enzymes that modify HSPG (Baeg et al., 2001; Lin and Perrimon, 2000). One school of thought suggests that HSPGs act as co-receptors on target cells (Fig. 5). However, cultured cells that lack Dally are still able to respond to Wg (Lum et al., 2003). Although redundancy with other HSPGs in these cell culture experiments is not excluded, it is possible that HSPGs act in other steps of Wg signaling, such

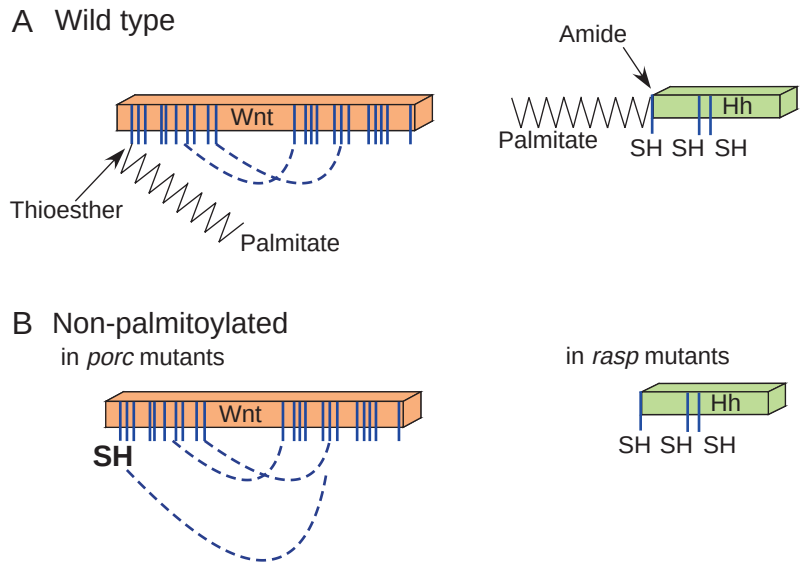

Fig. 4. Wnt and Hedgehog (Hh) acylation and the role of Porc and Rasp. (A) The palmitoylation of wild-type Wnt and Hh proteins. Wnt is palmitoylated through a thioesther to a cysteine. Dashed lines indicate the possibility of disulphide formation between other cysteines in Wnts. Hh is palmitoylated through an amide on the N-terminal cysteine. The two other cysteines in Hh are not disulphide linked and have free sulfhydryl (SH) groups. (B) In the absence of Porc, Wnt is not palmitoylated on the first cysteine. This cysteine then has a free SH group that interferes with disulphide formation between other cysteines. The resulting protein is midfolded and will not be secreted. In the absence of Rasp, the first cysteine in Hh is not palmitoylated but the number of free SH groups does not change and the Hh protein is still secreted.

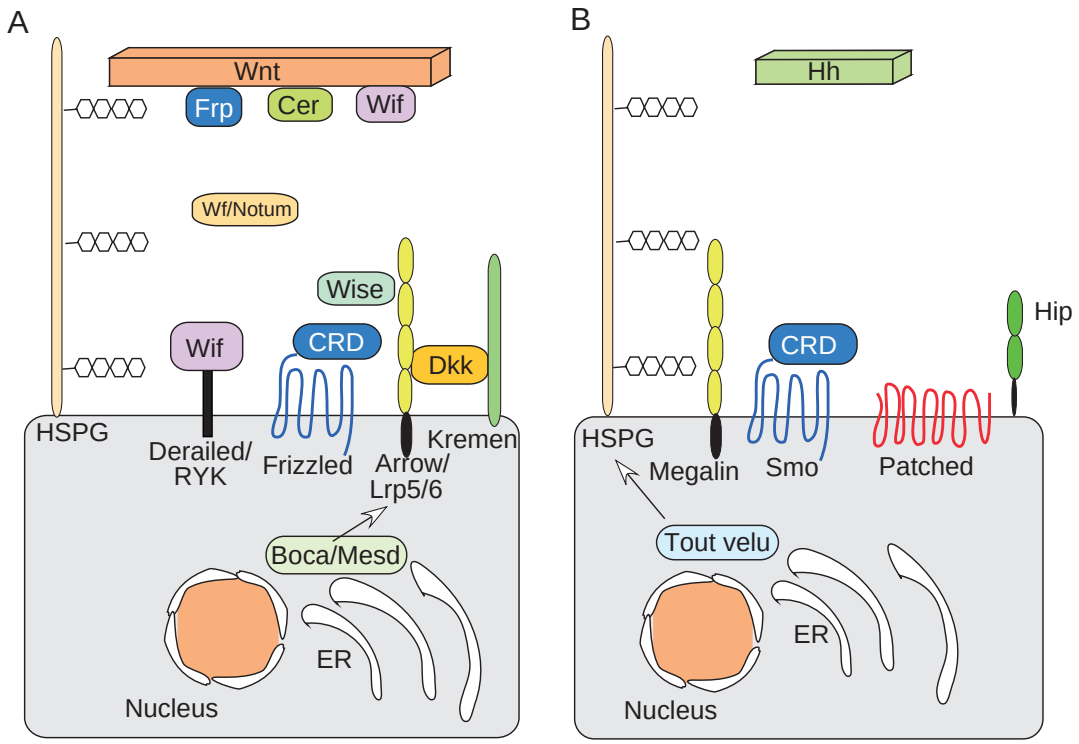

Fig. 5. Cell surface and secreted molecules implicated in Wnt and Hh signaling. (A) In vertebrates, Wnt proteins are inhibited by direct binding to either Frp (Frizzled-related protein), Cerberus (Cer) or Wnt inhibitory factor (Wif). Frp is similar in sequence to the cysteine-rich domain (CRD) of Frizzled, one of the Wnt receptors. The Wnt inhibitors Dickkopf (Dkk) and Wise bind to the Wnt co-receptors Arrow and Lrp (LDL receptor-related protein). Dkk also interacts with Kremen to downregulate these molecules from the surface. Wf/Notum is an extracellular Wingless inhibitor found in *Drosophila*. In *Drosophila*, it is also shown that a Wnt can bind to the tyrosine kinase receptor Derailed (RYK in mammals). This receptor has a domain similar to Wif. Heparin-sulfated forms of proteoglycans (HSPG) are also involved in Wnt reception or transport. Boca/Mesd is specifically required for the transport of Arrow/Lrp in the ER. (B) Hh signaling requires the membrane receptors Smoothened (Smo) and Patched and is modulated by HSPGs, which are possibly modified by Tout velu. Megalin, a protein related to Arrow/Lrp has been found to bind to Hh, but only in vertebrates. The Hedgehog inhibitory protein Hip binds to Hh and inhibits its function, but it has only been found in vertebrates. See text for a further explanation of these molecules.

as in the transport of Wg, and that they are not required for reception.

With respect to Hh, compelling evidence is emerging for a role of HSPGs in its transport, provided by the phenotype of the *tout-velu* (*ttv*) fly mutant (The et al., 1999). This gene encodes an enzyme involved in HSPG synthesis in *Drosophila* and its absence in flies interferes with Hh signaling. Most intriguingly, Hh protein is unable to move through cells that lack *ttv* (The et al., 1999), although transport of the cholesterol-free HhN is not affected. This observation has been interpreted as evidence that Ttv specifically promotes the transport of cholesterol-modified Hh, but it might well be that palmitoylation is equally relevant in the role of Ttv in Hh transport. If so, one might expect that the *ttv* mutant would affect Wnt signaling as well, something that remains to be investigated.

Reception of Wnt and Hh signals

Cells employ multiple receptors to receive instructions from Wnt and Hh signals, in complex and little understood configurations (Fig. 5). Remarkably, the Frizzled (Fz) receptors for Wnts (Bhanot et al., 1996) are related to the Smoothened (Smo) protein that is necessary for Hh signaling (Alcedo et al., 1996; van den Heuvel and Ingham, 1996). Both receptors have seven transmembrane domains and a long N-terminal extension called a CRD (cysteine-rich domain). As a group, these molecules are more closely related to each other than they are to the other families of serpentine receptors, of which there are many.

Although Fz and Smo are related to each other, it should be stressed that the actual mechanisms of activation of Fz and Smo are fundamentally different. Smo is not thought to interact with an extracellular ligand, whereas Wnt proteins bind directly to the CRD of Fz (Bhanot et al., 1996; Dann et al., 2001; Hsieh et al., 1999b). In vertebrates, little is known about the specificity that exists between Wnt ligands and receptors, but, in *Drosophila*, affinity between genetically matched pairs of Wnts and Fzs is high (Rulifson et al., 2000; Wu and Nusse, 2002).

The way in which Hh engages with its receptor has been subject to debate, but a consensus has emerged in which Hh binds to Patched (Ptc) (Chen and Struhl, 1996; Ingham et al., 1991; Stone et al., 1996), a 12-pass transmembrane protein related to Disp. As a consequence of this interaction, Smo is activated, or, to be more precise, is released from an inhibitory activity that is exerted by Ptc when Ptc is not engaged by ligand. In other words, the negative interaction between Ptc and Smo is relieved by binding of Hh to Ptc, which turns Ptc

activity off. Inhibition of Smo by Ptc may not by direct binding, but rather by a catalytic activity of Ptc (Taipale et al., 2002). As Smo can be both activated (Frank-Kamenetsky et al., 2002) and inhibited (Taipale et al., 2000) by small molecules, it has been suggested that Ptc might act as the transporter of an endogenous small compound that interacts with Smo (Taipale et al., 2002). Smo can be activated to a constitutive, i.e. Ptc-independent, state by specific point mutations found in tumors (Taipale et al., 2000; Xie et al., 1998), and it is thought that these mutant forms of Smo are refractory to inhibition by small molecules (Frank-Kamenetsky et al., 2002).

In the context of comparing Smo and Fz in this review, it may be useful to include some additional observations on these molecules, including some unpublished results from our own lab. First, there is no evidence that Smo can bind a Wnt-like ligand. There are no known genetic interactions between Wnt genes and *smo*, and, in direct binding assays, the Smo-CRD failed to interact with any of the *Drosophila* Wnt proteins tested (Wu and Nusse, 2002). Second, the small molecules that modulate Smo activity, including cyclopamine (Cooper et al., 1998), do not seem to have any effect on Wnt-Frizzled signaling in intact animals, in particular in mice. This can be deduced from the phenotype generated by these compounds in vivo (Frank-Kamenetsky et al., 2002). Tissues that are affected by the small molecule inhibitors include those malformed by absence of Hh (Cooper et al., 1998; Frank-Kamenetsky et al., 2002), but they do not resemble any Wnt or Frizzled mutant phenotypes. Third, the mutations that activate Smo in various cancers include a tryptophane to alanine mutation in the last transmembrane domain of Smo (Xie et al., 1998). Despite the conservation of that residue in Fz, mutating it in the same way does not active Fz to a ligand-independent state (M. Brink and R.N., unpublished). It seems therefore that Smo and Fz, despite their kinship, operate in different ways.

Other receptors and cell surface molecules

Adding to the complexity of Wnt and Hh reception is the existence of several other membrane molecules and alternative receptors for these signaling pathways (Fig. 5). Wnt signaling requires not only a functional Fz, but also the presence of a long, single-pass transmembrane molecule of the Lrp (LDL receptor-related protein) family, which is encoded by *arrow* in *Drosophila* (Wehrli et al., 2000) and by *Lrp5* or *Lrp6* in vertebrates (Pinson et al., 2000; Tamai et al., 2000). It has been proposed (Tamai et al., 2000), but not always confirmed (Wu and Nusse, 2002), that Wnt molecules can also bind to LRP and form a trimeric complex with a Frizzled. The cytoplasmic tail of Lrp may interact directly with Axin, one of the downstream components in Wnt signaling (Mao et al., 2001; Tolwinski et al., 2003). The specificity of Lrp5/Lrp6/Arrow in the Wnt pathway is further illustrated by the identification, both in flies and in mice, of genes that are required as ER chaperones for transport and folding for Lrps. The chaperone genes, *boca* (Culi and Mann, 2003) and *Mesd* (Hsieh et al., 2003) have phenotypes very similar to Wnt pathway components (Fig. 5).

In mammals, but not in *Drosophila*, a protein called megalin may control the endocytic uptake of the Hh protein by acting as a direct binding partner (McCarthy et al., 2002). Moreover, mouse embryos lacking *megalin* are phenotypically similar to Hh mutants. Megalin is an LDL receptor-related protein (it is also called Lrp2), and is therefore related to Lrp5 and Lrp6, the Wnt interacting members of the family.

A recent discovery in *Drosophila* has presented compelling evidence that a different type of receptor for Wnt might exist. The guidance of axons in the CNS is regulated by a member of the Wnt family, DWnt5 (Wnt5 – FlyBase). *Drosophila* embryos mutant for DWnt5 are similar to those lacking the transmembrane tyrosine kinase Derailed. Indeed, the DWnt5 protein can bind to the Derailed cell-external domain (Yoshikawa et al., 2003). This domain contains a Wnt Inhibitory Factor (Wif) domain, which has previously been shown to interact with Wnt molecules (Fig. 5) (Hsieh et al., 1999a). There is specificity of interaction between Derailed and Wnts, as is shown by the inability of the Wg protein to bind to Derailed (Yoshikawa et al., 2003). It might also be relevant that DWnt5 does not bind to any of the *Drosophila* Fz molecules (Wu and Nusse, 2002). How Derailed couples to the cytoplasmic components of signaling is not clear; the kinase domain seems to be dispensable for its function (Yoshikawa et al., 2001). It is quite intriguing to find a different mode of Wnt signaling that operates in the migration and positioning of cellular projections, rather than in the cell fate decisions that most Wnts control. One can speculate that other Wnts, including vertebrate Wnt5, might employ a similar mechanism, and there is indeed evidence for Wnts acting as axonal guidance molecules in vertebrates (Hall et al., 2000).

Extracellular inhibitors of Wnt and Hh signaling and feedback loops

Most, if not all, signaling pathways are subject to negative regulation or feedback control (Freeman, 2000), and Wnt and Hh signaling are no exceptions. In *Drosophila*, expression of several Frizzleds is controlled by Wg activity, in such a way that Wg signaling is either inhibited or facilitated by different receptor levels. Dfz2 (Fz2 – FlyBase), the highest affinity receptor for Wg, is downregulated by Wg signaling and this leads in turn to lowered levels of active Wg protein (Cadigan et al., 1998). Dfz3 (Fz3 – FlyBase), yet another but lower affinity Wg receptor, is induced by Wg signaling (Sato et al., 1999; Sivasankaran et al., 2000) although the consequences of this regulation seem to be minor as Dfz3 mutants have a limited phenotype, if any (Sato et al., 1999; Sivasankaran et al., 2000). Hh signaling leads to elevation of the expression of Ptc on the cell surface, which results in sequestration of the Hh ligand (Chen and Struhl, 1996). Effectively therefore, Hh and Wnt control their own distribution by manipulating the expression of their receptors. The complexity of these circuits of regulation is further illustrated by the upregulation of Smo by Hh, which occurs at the protein level (Denef et al., 2000; Zhu et al., 2003).

In vertebrates, the Hh signal controls Ptc expression (Goodrich et al., 1997), but it also induces the expression of a cell-surface protein, hedgehog inhibitory protein (HIP), which binds to and sequesters Hh (Fig. 5) (Chuang and McMahon, 1999). No *Drosophila* counterpart of HIP has been found and, in general, vertebrates seem to have acquired a much more sophisticated set of secreted Wnt or Hh molecules than have insects or worms (Fig. 5). For example, Wnt signals in vertebrates can be inhibited by no less than 5 different secreted factors (Fig. 5). Wif (Hsieh et al., 1999a), frizzled-related protein (Frp or Frzb) (Moon et al., 1997; Rattner et al., 1997)

and Cerberus (Piccolo et al., 1999) bind to Wnt molecules themselves. As an alternative way of blocking Wnt signaling, dickkopf (Dkk) (Glinka et al., 1998) and the Wnt-modulator-in-surface-ectoderm (Wise) protein (Itasaki et al., 2003) modulate Wnt signaling by interacting with Lrp5/Lrp6/arrow. The mechanism by which Dkk acts is unusual: it removes Lrp from the cell surface through the endocytic pathway, and it does so by interacting at the same time with Lrp and with Kremen, another cell surface molecule (Mao et al., 2002).

None of these Wnt inhibitors have been found in the fly genome. However, *Drosophila* contains a gene that inhibits Wg and that is induced by the Wg signal: it is called *wingful* (Gerlitz and Basler, 2002) or *Notum* (Giraldez et al., 2002), and it may encode a hydrolytic enzyme whose substrate is unknown. Possible vertebrate homologs remain to be characterized.

What is the evolutionary origin of Wnt and Hh signaling?

Given the similarities between Wnt and Hh signaling, it is tempting to speculate about the origin of these pathways and to examine whether they can be traced back to a common ancestral pathway in evolution. *C. elegans* contains 5 different Wnt genes and three β-catenins. Likewise, the primitive diploblast Hydra contains a bona-fide Wnt and a set of Wnt pathway genes (Hobmayer et al., 2000). *Dictyostelium* has no Wnt, but a β-catenin-like gene (called *aardvark*) (Grimson et al., 2000) is involved in morphogenesis, and there are clearly recognizable homologs of β-catenin in plants as well (Amador et al., 2001). It is possible, therefore, that an ancient β-catenin-based mechanism was present prior to the evolution of animals. By adding Wnt and Frizzleds, β-catenin activity became subject to control from other cells, a quintessential aspect of organized multicellular life.

Sequence analysis has not revealed Hh-like genes in *C. elegans* or Hydra (although the genome of the latter organism is not yet completely known). However, the structure of the Hh molecule itself is very similar to that of zinc hydrolases and other enzymes, including bacterial ones (Hall et al., 1995). This similarly has fuelled speculation that the Hh signaling system is derived from an ancient metabolic pathway (Taipale and Beachy, 2001).

There are further interesting parallels between bacterial processes and signaling in animals. The inner bacterial membrane contains several translocators with an overall 12-transmembrane topology. Some of these translocators serve as efflux pumps to clear toxic drugs from the cell. These pumps contain multiple subunits (Murakami et al., 2002), including a 12-transmembrane molecule of the resistance-nodulation-cell division (Rnd) family, a proton-driven molecular transporter. The overall organization of the Rnd proteins is similar to that of Ptc/Disp, and there is conservation of some essential amino acids between these molecules (Taipale et al., 2002). As mentioned earlier, Ptc may control the translocation of small molecules over membranes in mammalian cells (Taipale et al., 2002).

Another class of translocators in the bacterial membrane belongs to the ABC transporter family, ATP driven translocators. These molecules also have 12 transmembrane domains, but their topology and sequence appears to be different from the Rnd/Ptc family (Yakushi et al., 2000). However, the function of these ABC transporters is reminiscent of Disp: they translocate lipid-modified proteins over the inner membrane into the periplasmic space (Yakushi et al., 2000). Moreover, many bacterial lipoproteins carry palmitate as their lipid, covalently linked to a cysteine at the N terminus of the protein (Yakushi et al., 2000). Among the functions of bacterial lipoproteins is cell-to-cell communication, as exemplified by the Tgl protein in *Myxococcus* (Nudleman and Kaiser, personal communication). Hence, a signaling system based on lipid-modified proteins and specific membrane translocators is ancient, and may have been the founder of the Wnt and Hh signaling systems. By exploring the similarities between these pathways and how they operate in different organisms, we should be able to make considerable inroads into understanding their roles in development and disease.

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