ELSEVIER

Release and trafficking of lipid-linked morphogens

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Wnt and Hedgehog family proteins are secreted morphogens that act on surrounding cells to pattern many different tissues in both vertebrates and invertebrates. The discovery that these proteins are covalently linked to lipids has raised the puzzling problem of how they come to be released from cells and move through tissue. A synergistic combination of biochemical, cell biological and genetic approaches over the past several years is beginning to illuminate both the forms in which lipid-linked morphogens are released from cells and the variety of molecular and cell biological mechanisms that control their dispersal.

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Current Opinion in Genetics & Development 2006, **16**:17–22

This review comes from a themed issue on
Oncogenes and cell proliferation
Edited by Allan Balmain and Denise Montell

Available online 20th December 2005

0959-437X/$ – see front matter

DOI 10.1016/j.gde.2005.12.006

Introduction

Wnt and Hedgehog family proteins are secreted ligands that control both patterning and proliferation during normal development. The activation of their signal transduction pathways is a major contributing factor to oncogenesis [1,2]. Wnt and Hedgehog family proteins are covalently linked to lipid [3–5], which confers a high affinity for cell membranes. Despite this, the proteins are released and can act on cells distant from the source of production. In this review, I discuss recent progress in understanding the trafficking of these proteins and the resulting implications for tumorigenesis.

Secretion of lipid-linked morphogens

Release of Hedgehog as large multimers

Recent studies from several laboratories have suggested different mechanisms by which lipid-linked morphogens might be released from cells: by the formation of high molecular weight aggregates; in association with lipoprotein particles; or by incorporation into exovesicular carriers. Sonic Hedgehog that is released into the supernatant by cultured cell lines appears to be present in high molecular weight aggregates that depend on lipid modification for their formation [6,7••,8••]. It has been proposed that these represent Hedgehog multimers associating through interactions with their lipid moieties (Figure 1a). However, the nature of these aggregates and whether they might also contain other proteins is not completely clear.

Lipoprotein association of Wingless and Hedgehog

Biochemical studies in *Drosophila* have shown that both Wingless (a Wnt-1 homologue) and Hedgehog associate with lipoproteins *in vivo*. Although more than 90% of these proteins are membrane-associated, virtually all the non-membrane-associated fraction of these proteins is bound to the *Drosophila* lipoprotein Lipophorin. Furthermore, a variety of glycophosphoinositol-linked proteins can also be found on these particles [9••]. Lipoproteins consist of a phospholipid monolayer surrounding a core of esterified cholesterol and triglyceride, and they are scaffolded by one of a family of apolipoproteins [10,11]. Lipid anchors mediating attachment to the exoplasmic face of the membrane would also fit well into the outer phospholipid monolayer of lipoproteins (Figure 1b). Knockdown of Lipophorin shows that these particles are required for long-range, but not short-range, signaling activity of Wingless and Hedgehog [9••]. Although Wingless and Hedgehog are still found in receiving tissue of these mutants, their distribution is abnormal: Hedgehog accumulates in cells near the source of production, and the amount of Wingless found outside cells is reduced. This may suggest that these morphogens mediate long- and short-range signaling by different mechanisms, only one of which depends on lipoprotein association.

The presence of Wingless and Hedgehog on lipoprotein particles has intriguing implications for the function of low-density lipoprotein (LDL) family receptor proteins (LRPs). A variety of LRPs have been shown to function in both Wnt and Hedgehog signaling. Vertebrate LRP5 and LRP6 and their *Drosophila* homologue, Arrow, are required for Wnt and Wingless signaling [12]. In vertebrates, LRP1 interacts with Frizzled receptors, and negatively affects Wnt signaling [13]. Another LRP, Megalin, has been shown to internalize Sonic Hedgehog [14], and some of the phenotypes of *megalin* knockout mice are consistent with loss of Hedgehog signaling [15]. It will be interesting to investigate whether these receptors interact specifically with the lipoprotein-associated forms of Wnts and Hedgehogs.

Release of Hedgehog on vesicular particles

Sonic Hedgehog has been detected in extracellular vesicular structures called nodal vesicular particles (NVPs),

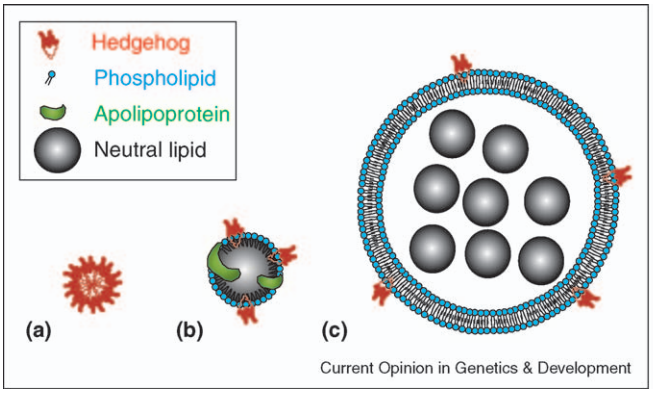
Glossary
7-pass transmembrane proteins: Proteins comprising seven transmembrane-domains. They often use heterodimeric G0proteins as secondary messengers.
Epistasis analysis: This technique determines the relative order — whether they operate upstream or downstream — of two gene products in a signaling pathway, by analyzing the phenotype of combinations of gain-of-function and loss-of-function mutations.

which are located on the surface of the ventral node during mouse embryonic development [16]. These particles also contain retinoic acid. Live imaging studies in which NVPs are labeled by diI (3,3′-dioctadecylindocarbocyanine) suggest that they are transported leftward across the node by cilia-dependent fluid flow, and that they are fragmented during the process. In the electron microscope, NVPs appear as multiple lipophilic granules surrounded by an outer membrane (Figure 1c) and some images suggest that they are generated from swellings in apical microvilli. Could the lipophilic granules inside NVPs correspond to lipoprotein particles? Their ultrastructural appearance is not dissimilar. Lipoprotein particles might provide an ideal environment for retinoic acid — in fact, the insect lipoprotein Lipophorin appears to transport hydrophobic molecules such as retinoids, juvenile hormone and phermone hydrocarbons [17–20]. Alternatively, these Hedgehog-containing particles may be completely different cell biological entities.

Transfer of morphogens through direct contact

Cells in tissue generate a variety of protrusions, enabling direct membrane contacts between widely separated cells. If lipid-linked morphogens were localized to such protrusions, they might be transferred to more distant cells by trans-endocytosis. In support of this idea, Patched-expressing tissue culture cells can internalize a transmembrane-domain-linked Hedgehog from adjacent cells [21]. Cells of the *Drosophila* wing imaginal disc construct long apical protrusions, termed cytonemes, that extend towards the Decapentaplegic (Dpp)-secreting cells at the anterio-posterior (A–P) boundary, and towards the Wingless-secreting cells at the dorso-ventral (D–V) boundary [22••]. Cytonemes contain vesicles bearing the Dpp receptor Thickveins (Tkv), suggesting that Dpp internalization may occur there [22••]. It is unknown whether direct contact between cytonemes and Wingless-producing cells might enable Wingless uptake. Imaginal disc epithelia also produce numerous basal filopodia [22••,23]; their role in morphogen transfer should also be investigated.

Figure 1



Proposed forms for secreted Hedgehog. **(a)** Hedgehog forms multimers by aggregation through lipid anchors. **(b)** Hedgehog associates with the phospholipid monolayer of lipoprotein particles through its lipid anchors. **(c)** Hedgehog buds from microvilli on membranous vesicles containing lipophilic granules.

These experiments have suggested that Hedgehog may be released from the cell membrane in three different ways: as high molecular weight aggregates; on lipoprotein particles; and as lipophilic granules within membranous parcels (Figure 1). It also seems plausible, although it has not been demonstrated, that direct, protrusion-mediated physical contact could release lipid-linked morphogens to other cells. Although some of the apparently dissimilar characteristics of released Hedgehog in these studies may resemble the alternative descriptions of the elephant provided by the proverbial blind observers — one, feeling the trunk, thought it was a snake; another, feeling the leg, thought it was a tree — the ability to present morphogens in different physical contexts could certainly expand the different types of signals transmitted to receiving tissue. Morphogens that are present on particles or on the plasma membrane might be accompanied by other proteins that alter their function. For example, other constituents of lipoprotein particles, such as glycophosphoinositol-linked proteins or even apolipophorin, might help to co-activate or cross-link a different set of receptors than do more homogeneous morphogen aggregates. Yet different consequences might result from presenting morphogens in the context of either plasma membrane or exovesicle proteins. The release of Hedgehog in a variety of forms with different inherent ranges might generate positional information as effectively as a morphogen gradient, and would also have the potential to increase tissue-specificity of signaling readouts.

Trafficking of Wnt and Hedgehog proteins in receiving tissue

Once secreted, *Drosophila* Wingless and Hedgehog move in an extracellular manner, and their range is limited by endocytosis [24••,25,26••,27•,28•]. A variety of other mechanisms promote, rather than inhibit, the spread of these morphogens.

Hedgehog transport by cilia-dependent currents

Currents generated by nodal cilia appear to be important for the dispersal of Hedgehog on NVPs. The structure and function of cilia that generate leftward flow in the ventral node depend on the activity of the intraflagellar

transport proteins. These proteins were first identified in single-celled algae, in which they are needed for the growth and maintenance of flagella [29]. When ciliary function is disturbed by mutation of genes encoding the intraflagellar transport proteins, Hedgehog and RA-containing NVPs are still released but do not flow to the left side of the node [16]. Interestingly, work from Kathryn Anderson's laboratory has shown that mutating any of the intraflagellar transport genes disturbs Hedgehog signaling in the neural tube [30•,31]. The convergence of these two studies might suggest that Hedgehog could be transported by a similar mechanism in this tissue. Indeed, the Huttner laboratory has demonstrated that the lumen of the neural tube contains exovesicular membranous particles that are enriched with Prominin and appear to be generated from microvilli [32]. Nevertheless, epistasis analyses (see Glossary) of mutations in the Hedgehog pathway and of intraflagellar transport mutants are not consistent with this model [31]. These studies suggest that intraflagellar transport mutants affect signaling either downstream of or in parallel to Smoothened, a 7-pass transmembrane protein (see Glossary) that mediates Hedgehog signaling. Furthermore, intraflagellar transport proteins appear to be required to generate both the activator and the repressor forms of Gli protein [31,33•,34••], and Smoothened must localize to the primary cilium to signal effectively [35••]. In *Drosophila* epithelial cells, which do not construct primary cilia, the complexes that control processing of the Gli homolog Cubitus Interruptus nevertheless localize to microtubules [36], supporting the idea that downstream signaling events occur on a microtubule-based scaffold. It is not clear whether cilia might have an additional function in distributing Hedgehog-containing particles in the vertebrate neural tube.

Heparan sulfate proteoglycans regulate morphogen dispersal

Heparan sulfate proteoglycans (HSPGs) are required for a wide variety of signaling events in vertebrates and invertebrates. Often, they act as ligand co-receptors, but recent studies in *Drosophila* have suggested that they also influence morphogen trafficking: they stabilize Wingless and Hedgehog and disperse them through tissue. Mutations that block the synthesis of heparan sulfate prevent the accumulation of Wingless and Hedgehog in both producing and receiving tissue of *Drosophila* wing imaginal discs [37,38•,39••]. In producing cells, this may reflect decreased stability of the protein. However, the situation in receiving tissue is more complex. Morphogens not only fail to accumulate in receiving tissue but build up to abnormally high levels in adjacent wild type producing cells [39••]. This suggests strongly that receiving cells missing heparan sulfate cannot acquire morphogen from their neighbors and that it is actively required for the transfer of morphogen from cell to cell.

Which specific HSPGs might be responsible for these effects? The glycophosphoinositol-linked HSPGs (glypicans) Dally and Dlp may contribute to Hedgehog dispersal in the wing disc, although the effects of even double mutants on the spread of Hedgehog protein are much milder than those produced by blocking heparan sulfate synthesis [22••]. It has been suggested that the Syndecan homologue, Trol, promotes Hedgehog dispersal in the brain [40], and it might be an interesting candidate for examination in the imaginal discs.

Recent studies have suggested that Dlp is important for long-range dispersal of Wingless in imaginal discs [26••,41••–44••]. In Dlp mutant wings, the Wingless gradient is steeper. Consistent with this, in Dlp mutants, long-range signaling is reduced, and short-range signaling is increased [43••,44••]. By contrast, Dally appears to be important for short-range Wingless signaling. This may in part reflect the different expression patterns of these two genes: Dally-like expression is reduced near Wingless-producing cells, whereas that of Dally is elevated. Nevertheless, their overexpression phenotypes suggest that the two do not function identically [41••]. Although the glypicans appear to be important for Wingless trafficking, the consequences of removing these proteins are not identical to those of blocking heparan sulfate synthesis. In particular, Wingless is depleted rather than elevated in wild type cells adjacent to Dally mutant clones, and Dally-like clones cause very modest accumulation of Wingless in adjacent wild type tissue. This may suggest that other HSPGs also influence Wingless trafficking; alternatively, blocking heparan sulfate synthesis could alter rather than abrogate the function of HSPGs.

How might heparan sulfate assist in the spread of lipid-linked morphogens? One proposal is that heparan sulfate restricts the diffusion of morphogens to the epithelial layer, effectively increasing their concentration [41••]. Another possibility is suggested by the observation that Dlp, which normally causes Wg to accumulate on cells that express it, can be released from its glycophosphoinositol anchor by the enzyme Notum; if morphogens were released with Dlp they might spread over longer distances [42••]. Other evidence suggests that HSPGs might act to prevent morphogen endocytosis, increasing the distance over which they spread before being degraded [26••,44••]. In support of this, Dlp is not required for the stabilization or spread of Wingless through the disc epithelium if endocytosis is prevented [26••]. Finally, the different forms in which morphogens can be released raise the possibility that HSPGs could affect dispersal and stability by more complex mechanisms — perhaps by altering the trafficking of the particle on which morphogens travel, or by promoting protrusion outgrowth. The synergistic combination of biochemistry, cell biology and genetics has produced rapid progress in

the past several years, and we are poised on the verge of understanding these important problems.

Implications for oncogenesis

Although Hedgehogs and Wnts control patterning and proliferation during embryonic development, de-regulation of these signaling pathways can also promote uncontrolled proliferation and metastasis [45–51]. For some cancers, the transformed phenotype is caused not by mutation of signal transduction pathway components but by over-production of the morphogen itself. Tumors of the pancreas, stomach and esophagus constitutively synthesize Sonic Hedgehog, which is required for continued proliferation [52••,53••]. The synthesis of Wnt family proteins is upregulated in human colon cancer and melanoma [54,55], and anti-Wnt-2 antibody inhibits the growth of melanoma cells [56••]. Recent progress in understanding how Wnt and Hedgehog family proteins are released suggests new intervention strategies for such tumors, some of which are amongst the most recalcitrant to current therapies. The fact that release of Hedgehog on NVPs appears to require fibroblast growth factor signaling suggests that inhibitors of this pathway might be tested on Hedgehog-secreting tumors. Furthermore, the observation that Wingless and Hedgehog can be released on lipoprotein particles raises the possibility that lipoprotein-lowering drugs, such as statins or inhibitors of microsomal triglyceride transfer protein, may block the progression of tumors that secrete these morphogens. Interestingly, statins appear to inhibit proliferation and survival of a broad variety of tumors [57,58].

The wide-ranging effects of statins on different types of tumor cells are not completely understood and may have many causes. Statins inhibit the production of mevalonate by hydroxymethylglutaryl co-enzyme A reductase. Mevalonate is required for synthesis of sterols and isoprenoids, as well as for synthesis of dolichols and ubiquinones. Small GTPases of the Ras and Rho families are often activated in transformed cells, and their membrane localization and activity depends on isoprenylation; in some cases, the tumor inhibitory effect of statins appears to result from blocking isoprenylation of these GTPases [59–62].

Other evidence suggests that the effect of statins of cholesterol synthesis has the potential to regulate proliferation. Although vertebrate cells can obtain sterols either by LDL uptake or by endogenous synthesis, insects are sterol auxotrophs. Removal of sterol and other lipids from the diet of *Drosophila* during the rapid proliferation that occurs in larval stages results in growth arrest and miniature adults [9••]. In vertebrate cells deprived of LDL, distal cholesterol biosynthesis inhibitors that enable the production of isoprenoids, dolichol and ubiquinone cause cell cycle arrest at the G_2–M transition [63••]. The arrest can be rescued by specific addition of cholesterol, but not other sterols [64,65]. These studies suggest there may be regulatory pathways linking sterol availability to proliferation. In this context, it is intriguing that Hedgehog and Wingless bind to and are internalized with the lipoprotein particles that deliver sterol to cells. It would be interesting to explore how the presence of these morphogens on Lipoprotein particles affects particle internalization, trafficking and unloading. Clearly, progress in understanding how these morphogens link tissue growth to patterning during normal development will continue to have broad implications for the origin and treatment of tumors.

Acknowledgements

I gratefully acknowledge the support of the Max Planck Gesellschaft and colleagues at the Max Planck Institute for Molecular Cell Biology and Genetics in Dresden.

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