A conserved mechanism of Hedgehog gradient formation by lipid modifications

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Abstract

Members of the Hedgehog (Hh) family of proteins are conserved morphogens that modulate cell fates in target tissues in different developmental systems. Dysregulation of Hh signaling results in a wide range of human diseases. The mature Hh is dually lipid-modified, with palmitate at the N-terminus and cholesterol at the C-terminus. The lipid modifications are essential to the proper secretion and spreading of the morphogen throughout the extracellular matrix, interacting with heparan sulfate proteoglycans. However, the role of lipid modifications in regulating Hh range and activity remains controversial. Here, we aim to resolve this issue by providing a model that is congruent with current and past literatures. We propose that the cholesterol moiety functions to restrict the dilution and deregulated spread of the morphogen in the extracellular space.

Introduction

A key issue in developmental biology is how cells in a developing field acquire the positional information that will determine their fate. Secreted members of the Hedgehog (Hh) family are essential signaling molecules controlling growth and patterning in both vertebrates and invertebrates (1). Hh proteins are considered to act as morphogens that can spread from localized sites of production to specify a diverse array of cell fates, ranging from segmental patterns in *Drosophila* larva cuticle to neurons in the vertebrate neural tube, in a concentration-dependent manner (1, 2). The mature Hh is synthesized as a precursor protein that undergoes a series of postranslational modifications, leading to covalent attachment of a cholesterol moiety at its carboxyl-terminus and palmitic acid at its amino-terminus (Box1). The cholesterol moiety of Hh has been shown to tightly associate with the cell membrane (3) and a specific cellular mechanism has to be deployed to facilitate the release of the highly lipidated Hh from

its source. The twelve-pass transmembrane protein, Dispatched (Disp), appears to fulfill this requirement (4) as it is only required for the release of cholesterol-modified Hh (Box 2). Thus, the cholesterol moiety of Hh is necessarily coupled to Disp function for the regulated release of Hh.

The extracellular spreading of Hh is a highly regulated process and is a critical determinant of morphogen gradient. The hydrophobic nature of Hh lipid modifications would be predicted to have a significant effect on the shape and range of activity gradients. Indeed, expression of different forms of Hh that lack either cholesterol moiety (Hh-N or Shh-N) or palmitic acid (HhC85S or ShhC25S) in several animal models led to profound alterations in spreading and signaling properties of Hh. The loss of palmitoylation resulted in strong developmental defects, indicating that palmitate modification is required for Hh activity (5-9). The loss of activity in HhC85S may be associated with the observation that it is not internalized by its receptor Patched in *Drosophila* imaginal disc epithelia (10), although in vitro experiments indicate that purified ShhC24S is capable of binding to Patched as efficiently as lipidated Hh (11). In contrast, the cholesterol moiety does not appear to be much necessary for Hh activity (12). Instead, it appears to affect the capacity of Hh to signal by modulating its distribution. However, its role in regulating Hh spread and signaling in *Drosophila* and vertebrates is controversial. The major issue that needs to be resolved is whether the cholesterol moiety promotes or restricts Hh spreading to generate a defined activity gradient. Here, we provide a unifying model of the functions of the cholesterol moiety that is congruent with current and past literatures.

Cholesterol modification in the regulation of *Drosophila* Hh gradient

Although the Hh signal was first described as a morphogen in the segmental patterning of the *Drosophila* larval cuticle (13), the adult wing pattern offers a unique readout of Hh signaling. The wing disc consists of an epithelial sack with a thick columnar pseudostratified epithelium on one side and squamous epithelium, called the peripodial membrane, on the other. The apical surfaces of these two epithelia are oriented towards the disc lumen. Two populations of cells with different cell adhesion affinities divide the epithelium of columnar cells into posterior (P) and anterior (A) cells. Hh is produced by the posterior compartment cells and spreads into the anterior compartment (14, 15). Hh forms a gradient over a diameter of about twelve cells in the anterior/posterior compartment border and activates a series of target genes in a concentration-dependent manner. Examples of target genes that respond to high threshold levels of Hh are *engrailed (en)* and *patched (ptc)*, while *decapentaplegic (dpp)*, *collier (col)*, *cubitus interruptus (ci)* and *iroquois (iro)* respond to low threshold levels of Hh (reviewed in (16)). Three recent reports (10, 17, 18), analyzing the effect of lipid modification on Hh spreading, agree that lack of cholesterol leads to a reduction in the activation of high threshold targets with a concomitant reduction of the range over which these targets are activated. However, there is disagreement on whether Hh-N activates the low threshold targets over an extended or reduced range. Callejo et al. (10) and Dawber et al. (17) have shown that Hh-N spreads and activates the low threshold Hh responses across many more cell diameters than wildtype Hh, presumably at the expense of reduced signaling activity near the source. In contrast, Gallet et al. (18) described that Hh-N exhibited a reduction in the range over which it activates both high and low threshold Hh responses, suggesting that cholesterol modification of Hh is required for its long-range activity.

To analyze the cause of the discrepancy, we have to consider the experimental

method used to generate Hh-expressing clones. In all three reports, the various forms of Hh were expressed in flies using the Gal4/UAS system, which allows expression of UAS transgenes (Hh, Hh-N or HhC85S) in ectopic Gal4 clones (19). To visualize the gradient, Callejo et al. used the GFP-tagged forms of Hh, but experiments were also performed with untagged forms, which gave identical results (10). The expression level of Hh will depend on the number of cells in the ectopic clone (clone size) and/or the level of ectopic protein induced after Gal4 induction. Also pertinent to this discussion is the observation that Hh-N has a decreased affinity for plasma membrane (18) and did not interact properly either with the Ptc receptor (10) or with extracellular components such as heparan sulfate proteoglycan (HSPG) (20) or Shifted (21, 22). The inability of Hh-N to efficiently interact with extracellular components would, therefore, lead to unregulated Hh-N spreading and dilution. This may explain the apparent reduction of high threshold Hh-N responses and the range over which these target genes are normally activated. On the other hand, the induction of low threshold Hh response genes at a distance is sensitive to Hh expression level (i.e, clone size) at the source. In small clones, as in studies by Gallet et al., Hh-N concentration would be below the threshold levels to exert long-range signaling activity and to be detected by antibody staining (18). In large clones, however, the higher level of Hh-N production would permit detection of long-range Hh-N spreading and the activation of low threshold responses (10) (17). This dilution of Hh-N into the extracellular space is more apparent when Hh-N is expressed only in the peripodial membrane (10, 18). Under this experimental condition, Hh-N is presumably secreted into the lumen to activate low threshold target genes in the subjacent epithelium as it accumulates near its apical surface. Therefore, from the three recent reports and from a previous one (4), we can deduce that restricting Hh spreading by the cholesterol moiety prevents Hh dilution, permitting both correct Hh reception and precise

Hh spreading through the epithelial surface. The critical role of the interaction of lipid-modified Hh with extracellular components in limiting Hh dilution has also been suggested in a mathematical model for Hh gradient formation (23).

Cholesterol modification in regulating the Shh gradient

Analogous to the *Drosophila* wing imaginal disc, the vertebrate limb bud has become an ideal model system to elucidate morphogen gradient action. The anterior-posterior (A/P) asymmetry of digit patterns is controlled by a group of specialized mesodermal cells located at the posterior margin of the limb bud referred to as the zone of polarizing activity (ZPA). Cells in the ZPA secrete Shh which functions as a classic morphogen in establishing the A/P polarity of the limb (24). It is thought that long-range extracellular movement of Shh in the limb bud depends on its cholesterol moiety, as the distribution of ShhN and its pathway activity were restricted to cells at or near the ZPA (25). However, embryos in the study also showed significantly lower ShhN expression level than wildtype, suggesting that RNA stability may have been compromised. These embryos expressed ShhN from a gene targeted allele that harbored a stop codon at the cleavage site, thus, it is possible that nonsense-mediated RNA decay due to the generation of a long 3' untranslated region, may have contributed to reduced Shh RNA level in the ZPA, as has been recently demonstrated for the *immunoglobulin- μ* gene (26).

To circumvent potential setbacks associated with RNA instability, Li et al. recently generated mice harboring the ShhN allele without the introduction of a premature stop codon at the cleavage site (27). The expression of ShhN was achieved by Cre-mediated excision of the sequence downstream of the cleavage site, which is necessary for Shh processing and cholesterol modification. In contrast to the earlier study, Li et al. found that ShhN has the propensity to travel far from the ZPA to elicit ectopic pathway activation in the anterior margin

of limb buds that exclusively expressed ShhN. Interestingly, the extended range of ShhN movement across the A/P axis of the limb bud was accompanied by appreciable reduction of local ShhN level, suggesting that the cholesterol moiety is required to increase local Shh concentration, thus preventing the dilution of Shh ligand near its source as has been predicted from mathematical modelling of Shh dynamics in the neural tube (23). This may explain the apparent reduction in the expression of Shh target genes within its normal signaling range in limb buds exclusively expressing ShhN.

It is clear that the extended range of ShhN possesses signalling activity even at a low concentration as it has the ability to elicit ectopic Shh pathway activation in the anterior limb bud. However, the extent to which ShhN activity is interpreted by target tissues as functional readouts is likely context dependent. For example, Shh pathway activation in the anterior limb bud mesoderm by ShhN occurs early and is reinforced by positive Shh-Fgf feedback loop (28). By contrast, activation of ectopic Shh target genes in the dorsal forebrain neuroepithelium occurs much later during development, presumably due to a requirement for low level ShhN to accumulate to an effective concentration threshold (X. Huang, Y. Litingtung and C. Chiang, unpublished data), similar to that observed in *Drosophila* when Hh-N is expressed in the peripodial epithelium (10, 18). Based on this finding and recent *Drosophila* studies, the emerging theme is that the cholesterol moiety functions to generate a steep Hh gradient across a morphogenetic field by restricting Hh dilution and unregulated spreading (Fig. 2).

At least two models that are not mutually exclusive could be envisioned in which the cholesterol moiety functions to increase local Shh concentration and prevents unregulated Shh spreading and dilution. First, multimeric forms of Shh may function to concentrate Shh locally. Fractionation studies of the supernatant of Shh (or Hh)-expressing cells showed that lipid-

modified Hh participates in high molecular weight structures that likely represent multimeric complexes and cholesterol appears to mediate this multimerisation (9, 10, 18, 29, 30). The lipid moieties are thought to be embedded in the core of these complexes, analogous to micelles, thus concentrating Shh locally. Likewise, cholesterol may stabilize Hh interaction with the extracellular matrix molecule, HSPG, as has been proposed in *Drosophila* (10, 21, 22). However, the role of HSPG in vertebrate Hh signaling has not been well established. There are three members of exotosin (EXT) class of enzymes involved in heparan sulfate biosynthesis. Targeted deletion of *Ext1* or *Ext2* in mice does not affect early patterning events mediated by Shh. However, expanded Ihh signaling in the growth plate of developing bones was observed in mice carrying a viable *Ext1* hypomorphic allele (31), consistent with the proposed role of HSPG in regulating Hh spreading range. Additionally, Misexpression of Sulfatase 1 (Sulf1), a secreted enzyme that modulates the sulfation state of heparan sulfate proteoglycans, in the neural tube can enhance and extend the range of detectable Shh protein level in the ventral neural epithelium at the onset of oligodendrogenesis, suggesting that Sulf1 may facilitate Shh-induced oligodendrocyte specification by augmenting Shh binding to HSPGs (32).

Concluding remarks and future directions

The data suggest that the mechanisms of Hh secretion and spreading is largely conserved throughout evolution from *Drosophila* to vertebrates, in spite of some functional divergences in the intracellular signaling that Hh elicits in the receiving cells. It is evident that cholesterol modification of Hh is essential to restrict dilution and deregulated spreading of the morphogen through the extracellular environment. In its absence, Hh spreads far from the source and has capacity to elicit ectopic low threshold pathway activation that depends on Hh expression level, tissue types and tissue responsiveness. Similar logic may also explain the paradoxical

observation that Shh that lacks palmitate modification is localized to its site of synthesis in several embryonic tissues (9). Analysis of Shh distribution in early stage embryos where ShhC25S transcript is not appreciably compromised may resolve this issue.

While the HSPGs appear to play a key role in regulating Hh movement, it is unclear as to how these extracellular proteins modulate Hh spreading, stability and signaling specificity. Are HSPGs and the enzymes involved in their production developmentally regulated? Does the degree of sulfation on the HS chain contribute to binding specificity? Another unresolved issue is how Hh spreads through different tissues. Does Hh move through the apical or lateral part of the polarized epithelium? How is Hh movement different in non-polarized tissue such as the vertebrate limb mesoderm? Does Hh reception occur through the apical or basolateral plasma membrane? Do receptor and co-receptors have a specific location in the polarized plasma membrane? Since the rate of Hh secretion and reception influences the formation of the Hh gradient, many open questions about the roles of Disp and Ptc functions still remain. It is particularly intriguing that they both belong to the sterol-sensing domain (SSD) protein family (see box2), but one of these proteins causes the release of Hh while the action of the other is to sequester Hh. What is the role of the SSD? Does cholesterol bind directly to this domain as in the case of other SSD proteins such as Niemann-Pick C1 and SREBP cleavage-activating protein? How specific are the SSD domains of Ptc and Disp? New model systems with cell biological and biochemical advances should help to elucidate some of these questions.

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Box1: Lipid modifications of Hh signal.

The mature and signaling domain of Hh protein is located in the amino (N)-terminal portion of the precursor protein and it is generated through an internal autocleavage at a glycine (G) residue. The cleavage reaction is mediated by the carboxy terminal domain of the Hh precursor, which also serves to couple cholesterol covalently to the N-terminal product of the signaling domain (reviewed in (11)). A second lipid modification that incorporates palmitic acid occurs at the amino-terminal cysteine (C) exposed after signal peptide cleavage (32). Cholesterol modification does not appear to be required for palmitoylation as a detectable amount of HhN was palmitoylated (9). This acylation appears to be catalyzed by the product of the *sightless* gene, also designated *skinny hedgehog*, *centralmissing* or *rasp* (5-8), which encodes a protein belonging to the conserved family of enzymes called membrane bound O-acyltransferases (MBOAT). All metazoan species examined so far show the same biochemical and functional mechanisms of Hh maturation processes (reviewed in (11)).

Box2: Disp is dedicated for the release of lipid modified Hh.

Disp was initially discovered in *Drosophila* and functions in Hh secretion (4). Disp function is dispensable for secretion of Hh without lipid modifications ((4, 33) ; A. Callejo, N. Gorfinkiel and I. Guerrero, unpublished results). In *Disp* mutants, lipid-modified Hh accumulated in the plasma membrane of mutant cells (Burke et al). It has been hypothesized that Disp is involved in packaging lipid-modified Hh into freely diffusing aggregates (28). Mouse and human genomes contain two Disp homologs, and genetic studies in mice have indicated an essential role of one of these genes, *mDisp1*, in Hh signaling (34-36). As in *Drosophila*, *mDisp1* is required in Shh-producing cells and only for the lipid-modified form of Shh (26, 37). Similarly, inactivation of *Disp1* in zebrafish embryos also disrupted Hh signaling

(38).

The Disp protein, like the Hh receptor Patched (Ptc), is predicted to contain twelve transmembrane domains and a sterol-sensing domain (SSD) which is found in proteins involved in cholesterol homeostasis or cholesterol-linked signaling, such as 3-hydroxy-3-methyl-glutaryl coenzyme A reductase, Niemann-Pick C1 (NPC1), and SREBP cleavage-activating protein (SCAP) (reviewed in (39)). The mechanism by which Disp releases lipid-modified Hh is not known. Functional studies on other SSD proteins suggest that it may be important for localization of lipidated Hh to specific membrane domains for secretion (reviewed in (39)). In this context, it is interesting to note that the Disp ortholog in *C. elegans*, *che14*, is involved in the apical secretion of proteins needed for cuticle formation (40).

Figure Legends

Figure 1. Biogenesis of Hh processing and lipid modifications. (A) The N-terminal signaling domain is generated by removal of the C-terminal processing domain at an internal glycine residue. This cleavage is coupled to covalent modification by cholesterol at the exposed glycine. Palmitic acid is added to the N-terminal cysteine by a catalytic process involving Sightless, a membrane bound O-acyltransferase. (B) Hh variants lacking either cholesterol or palmitic acid. Hh-N contains an internal glycine-to-stop codon and with deletion of the C-terminal processing domain. Hh lacking palmitate (ShhC25S in mouse or HhC85S in *Drosophila*) contains a point mutation, replacing cysteine 25 or 85 to serine.

Figure 2. Model for cholesterol adduct function in regulating Hh activity gradient.

HhNp is secreted from the posterior *Drosophila* wing imaginal disc or vertebrate limb bud

and activates high threshold (++) targets near the source and low threshold targets (+) further away from the source. Note that in vertebrate limb buds, low and high threshold readouts of Shh activity may reflect the expression levels rather than distinct targets. In the absence of cholesterol, Hh-N does not interact with extracellular components such as HSPG efficiently and thus, it has propensity to spread far from the source and is capable of activating low threshold target genes at an extended range. However, if *Hh-N* expression level is low, the activation of low threshold target genes will not be detected, as Hh-N concentration would be below the threshold levels required to exert long-range signaling activity. Thus, the cholesterol moiety prevents Hh dilution by restricting Hh spreading, permitting both correct Hh reception and precise Hh spreading through the cell surface.