VIEWPOINT

Hedgehog Signaling: A Tale of Two Lipids

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Hedgehog proteins constitute one of the major classes of intercellular signals that control inductive interactions during animal development. These proteins undergo unusual lipid modifications and signal through an unconventional transmembrane protein receptor that is characterized by a sequence motif implicated in sterol sensing. Recent studies suggest that the lipid adducts regulate the range and potency of the signals, whereas the sterol-sensing domain is essential for receptor activity.

The role of inductive interactions in animal development has long been recognized, but only recently have the signaling molecules mediating these interactions been identified. Prominent among these are the Hedgehogs (Hh), a group of closely related secreted proteins encoded by a gene family originally discovered through the *Drosophila* segment polarity mutation *hedgehog*, but now well characterized from fish to humans (although notably absent from the nematode worm *Caenorhabditis elegans*) (*1*). Like other signals regulating embryonic development, members of the Hh family, including Sonic (Shh), Indian (Ihh), and Desert (Dhh) Hedgehog, are involved in a remarkably wide variety of processes, ranging from the control of left-right asymmetry of the body to the specification of individual cell types within the neural tube and brain. And as with other embryonic signals, the aberrant activity of Hh signaling underlies a number of human abnormalities and diseases, notably holoprosencephaly and basal cell carcinoma.

Among the various facets of Hh signaling uncovered over the past decade, its intimate association with lipids is particularly intriguing. Hh proteins undergo two lipid modifications during their maturation. The mature signaling forms of Hh proteins (termed Hh-Np) are covalently coupled to cholesterol at their COOH-terminal ends, an association that has not been characterized for any other protein to date and which occurs concomitantly with the autoproteolytic cleavage of the ~45-kD precursor from which they are derived (*2*). In addition, Hh proteins are palmitoylated on a highly conserved NH_2-terminal Cys residue (*3, 4*). This modification, unusual for a secreted protein, is catalyzed in *Drosophila* by a polytopic transmembrane acyl transferase encoded by the *skinny hedgehog/sightless* (*ski/sit*) gene (*4, 5*). Consistent with these lipid attachments, most of the Hh in *Drosophila* embryos associates with detergent-insoluble, sterol-rich membrane microdomains (*6*). These are analogous to the lipid rafts of mammalian cells that function as platforms for intracellular sorting and signal transduction.

Two additional observations imply the potential functional importance of the cholesterol coupling. Certain inhibitors of cholesterol biosynthesis, including the steroidal alkaloids jervine and cyclopamine, induce holoprosencephaly when administered to developing mammalian or avian embryos (*7*), phenocopying the effects of loss-of-function mutations in the *Shh* gene in mouse and human. In addition, the Hh receptor Patched contains a sterol-sensing domain (SSD), a motif originally identified in proteins that function in cholesterol homeostasis, such as SCAP (sterol response element binding protein cleavage activating protein) and HMGCoA (hydroxymethylglutaryl coenzyme A) reductase (*8*). These observations are consistent with an essential role for cholesterol in mediating the receptor-ligand interaction that activates the signaling pathway. Various lines of evidence, however, suggest a rather different role for cholesterol in Hh function.

That cholesterol coupling is dispensable for Hh activity soon became apparent when truncated forms of Hh were engineered comprising just the NH_2-terminal signaling portion of the protein, thus eliminating the need for autocleavage. These forms (referred to here as Hh-N) lack the cholesterol adduct typical of the endogenous proteins, yet they retain signaling activity when assayed either in vitro or in vivo (*9, 10*) and show affinities similar to that of Hh-Np for Ptc, the Hh-Np receptor (*3*). Why, then, should cholesterol inhibitors mimic the effects of loss of Shh activity? A key insight came with the discovery by Beachy and colleagues that cyclopamine and jervine have no effect on Hh processing but compromise the cellular response to Hh (*7*). This pointed the finger at some component of the Hh signal transduction mechanism as the target for these teratogens. Because Ptc is a constitutive repressor of the Hh pathway [the binding of Hh to Ptc is thought to activate the signaling pathway by blocking the repressive activity of Ptc (*11*)], Beachy and colleagues suggested, by analogy with other SSD-containing proteins, that Ptc activity might be promoted by a teratogen-induced block in sterol transport (*7*). However, Incardona *et al.* (*12*) could find no clear correlation between inhibition of cholesterol transport and the inhibition of the Hh response: Mutation of the NPC1 protein, which blocks cholesterol transport, does not compromise the response of cells to Hh. Instead, it was suggested that these compounds might act on a trafficking pathway that is shared by Hh signaling and cholesterol transport (*12*).

Suggestive evidence that intracellular trafficking plays an integral role in the Hh response has recently come from in vivo studies of the function of Rab23, a divergent member of the large family of Ras-like proteins that mediate vesicle fusion in the endocytic and secretory pathways. Surprisingly, Eggenschwiler and colleagues have shown that mutations in the mouse *rab23* gene cause highly specific defects in the embryonic central nervous system (CNS) reminiscent of those caused by inappropriate activation of the Hh pathway (*13*). These *rab23* mutations can suppress the effects of loss of Shh activity on CNS development, which implies that inactivation of Rab23 causes the ligand-independent activation of the Hh pathway. Thus, a mutation that presumably blocks a trafficking pathway has an effect on Hh signaling opposite to that caused by cholesterol synthesis inhibitors.

If trafficking is involved in the response of cells to Hh proteins, what are the transported cargos? One likely candidate is the G protein–coupled receptor–like protein Smoothened (Smo), which is essential for transduction of the Hh signal [reviewed in (*1*)]. Despite having structural similarity to receptors, Smo does not interact directly with Hh, but instead is repressed by Ptc. The binding of Hh to Ptc is assumed to relieve this repression and thus activate Smo. Although the mechanism of this regulation is currently obscure, recent studies have suggested that Ptc may control the subcellular localization of Smo: In cells responding to Hh or lacking Ptc activity, Smo is redistributed from unidentified intracellular compartments to the plasma membrane (*14*). Interestingly, mutations in the SSD of Ptc abolish its ability to repress Smo, and the mutant protein acquires a dominant negative activity, suggesting that it now acts to protect Smo from inactivation (*15, 16*). This could imply that shuttling Smo between different lipid microdomains regulates its activity. In this view, it is interesting that cyclopamine has been shown to act downstream

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of Ptc, directly inhibiting Smo activity (*17*).

If cholesterol is not required for Hh proteins to bind to and inactivate Ptc, what purpose, if any, does this adduct serve? As a potent inducing molecule, the range of action of any Hh protein needs to be precisely controlled if it is to elicit the appropriate cell types at the right time and in the right place. In many contexts, Hh proteins act at very short range, although in other instances (such as the ventral neural tube of vertebrate embryos), there is good evidence that the Shh protein traverses multiple cell diameters. Clearly, close association with the plasma membrane afforded by the lipid modification of Hh proteins could provide a mechanism for restricting the range of their activities. Consistent with this idea, the expression of unmodified Hh-N in the *Drosophila* wing primordium causes the aberrant and widespread activation of the Hh target gene *decapentaplegic* (*dpp*) many cell diameters away from the source of the protein (*18*). By contrast, cells expressing the same unmodified form of the protein, anchored in the membrane by a GPI (glycosylphosphatidylinositol) linkage, signal exclusively to their immediate neighbors (*19*). Yet even in the *Drosophila* wing, the endogenous lipid-modified Hh-Np normally traverses several cell diameters, implying that at least some of the protein must be released from the membranes of the cells in which it is produced. Genetic analysis has revealed that this release is mediated by Dispatched (Disp), a Ptc-related protein that is specifically required for the activity of endogenous processed Hh-Np (*18*). In the absence of Disp function, cells accumulate high levels of Hh-Np but fail to secrete it, resulting in the loss of Hh target gene expression in neighboring cells; Hh-N is completely immune to this requirement, being readily secreted by *disp* mutant cells (*18*).

Given that Disp overrides the cholesterol-mediated membrane anchoring of Hh-Np, how is the range of the signal controlled? Genetic studies in *Drosophila* have shown that a key restraining influence on the movement of Hh-Np is exerted by its receptor, Ptc. By manipulating Ptc activity in clones of cells in the wing imaginal disc, Chen and Struhl showed that Hh-Np traverses cells that lack Ptc, unhindered by its cholesterol adduct, before being bound and endocytosed by wild-type cells expressing the receptor (*20*). The *ptc* gene itself is a target of Hh activity, and thus Hh effectively promotes its own sequestration by up-regulating *ptc* transcription, a negative feedback mechanism that restrains its signaling range (*20*). Because Hh-N is immune from such sequestration, it must depend on the cholesterol adduct in Hh-Np. Yet, as pointed out above, lipid modification has no measurable effect on the affinity of Hh for Ptc (at least as assayed in vitro), and Hh-N appears to be endocytosed with Ptc in responding cells (*18*). So how does the unmodified Hh-N escape Ptc-mediated sequestration? An explanation may lie in the requirement for the function of the *tout velou* (*ttv*) gene for the movement of cholesterol-modified Hh-Np away from its source. Ttv is a type II transmembrane protein homologous to the vertebrate Ext proteins, which are implicated in heparan sulfate proteoglycan (HSPG) biosynthesis (*21*). Thus, *ttv* is thought to function by synthesizing a HSPG that binds Hh-Np in responding cells, facilitating its transfer between cells while at the same time possibly increasing its effective concentration for presentation to Ptc (*22*). Movement of Hh-N is, by contrast, independent of *ttv* function, which suggests that in the absence of cholesterol, Hh-N is not bound by the hypothetical HSPG. In this case, a major proportion of Hh-N might escape interaction with Ptc and hence diffuse freely through the extracellular space.

Paradoxically, recent studies have suggested that the absence of cholesterol from the mouse Shh protein inhibits its movement across its target field of cells without affecting its signaling activity (*23*). This finding might seem to imply that cholesterol modification has a rather different effect on the properties of the vertebrate protein. Yet it is consistent with the notion of an active process in which the cholesterol moiety is necessary for Shh to interact with the hypothetical Ext-dependent HSPG that mediates its movement. The finding that, in contrast to its *Drosophila* counterpart, unmodified Shh-N fails to diffuse away from its source may be explained by the expression in vertebrates of Hip1 (*24*), an additional Hh binding protein not found in *Drosophila*.

The role of the NH_2-terminal palmitoyl adduct in restricting the range of Hh is much less clear than the role of cholesterol in regulating its release and movement from secreting cells. In tissue culture cells, acylation of Shh-N is sufficient for membrane association of the protein (*3*), yet the widespread movement of *Drosophila* Hh-N when expressed in vivo would seem to argue against such an effect. Tissue culture studies suggest that acylation is very inefficient in the absence of autocleavage of the protein (*3*), which could explain the unfettered behavior of Hh-N in *Drosophila* imaginal discs. Notably, however, the phenotypic effects of Hh-N are abolished in *Drosophila* mutant for the *ski/sit* gene (*4, 5*). Although this finding implies that unmodified Hh-N is efficiently acylated in vivo, it may be that under the high expression levels used in these experiments, acylation of even a small fraction of the total Hh-N is sufficient to generate the observed signaling activity. The available data cannot distinguish between these possibilities, but they clearly indicate that palmitoylation is not sufficient to limit Hh movement in vivo. It is, however, possible that acylation of the cholesterol-modified form increases its membrane affinity.

A definitive resolution of this issue will require direct analysis of the distribution of the unacylated forms of the proteins. What is evident from the *Drosophila* data—and, indeed, from other earlier studies in vertebrate systems—is that, in contrast to cholesterol, the palmitoyl adduct plays a critical role in potentiating the activity of Hh proteins. In *Drosophila*, acylation is essential for Hh activity because it is completely abolished in animals mutant for the *ski/sit* gene (*4, 5*), and because mutation of the NH_2-terminal cysteine residue to which the palmitoyl group is coupled inactivates the protein (*4, 25*). Moreover, this mutated form of Hh acts as a competitive inhibitor of the endogenous protein, implying that it is normally secreted and can interact with Ptc (*25*).

The situation in vertebrates is broadly in line with these findings in *Drosophila*, but is more complex. Although early experiments had shown that bacterially expressed unmodified Shh-N induces motor neuron differentiation in explanted neural plate tissue, its activity in a separate cell line–based assay varied significantly according to the source of the protein. It was this variation that led to the discovery that Shh is palmitoylated (*3*), a modification that increases its potency by a factor of 30 in 10T1/2 mouse fibroblasts, but has no such effect on motor neuron induction. Similarly, in the mouse forebrain, acylation is essential for Shh-mediated induction of specific neuronal cell types (*26*), whereas in the limb, unacylated protein retains high levels of activity (*25*). Why there should be this difference in requirement for the palmitoyl adduct remains unclear, but it may reflect variation in the levels of activity required for different processes. Interestingly, recent studies by Taylor and colleagues have shown that the replacement of the NH_2-terminal Cys in Shh by a hydrophobic residue is itself sufficient to increase signaling activity (*27*). Thus, it is the hydrophobicity of the protein per se, rather than the specific nature of the palmitoyl moiety, that potentiates its activity. This fact argues against a role for acylation in promoting aggregation of the protein. Instead, these authors suggest that the hydrophobic moieties may mediate interaction with a general hydrophobic area, possibly membrane lipids.

It is clear that lipid modifications have profound effects on the properties of Hh proteins: The cholesterol moiety imposes controls on their secretion and movement, whereas palmitoylation potentiates their signaling activities. Exactly how these effects are mediated remains

to be determined. Does palmitoylation increase the effective concentration of Hh or increase its affinity for Ptc? Does cholesterol mediate the interaction of Hh-Np with HSPGs? And when and where does Disp act to regulate the release of Hh-Np from secreting cells? It also seems likely that the lipid environment plays a critical role in regulating the activity of the Ptc and Smo proteins, although, again, the details of these processes remain obscure. The studies reviewed here have given some tantalizing glimpses into the roles of lipids in these processes, but a great deal more analysis at the cellular and biochemical levels will be required before the picture can be completed.

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REVIEW

Location, Location, Location: Membrane Targeting Directed by PX Domains

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Phosphoinositide (PI)–binding domains play critical roles in the intracellular localization of a variety of cell-signaling proteins. The 120–amino acid Phox homology (PX) domain targets proteins to organelle membranes through interactions between two conserved basic motifs within the PX domain and specific PIs. The combination of protein-lipid and protein-protein interactions ensures the proper localization and regulation of PX domain–containing proteins. Upon proper localization, PX domain–containing proteins can then bind to additional proteins and execute their functions in a diverse set of biological pathways, including intracellular protein transport, cell growth and survival, cytoskeletal organization, and neutrophil defense.

With 30,000 to 40,000 genes potentially expressed in the human genome, cells face the difficult task of assembling these gene products into functional complexes and localizing them to appropriate sites. Of course, cells have developed a number of different strategies to deal with this problem, one of which is to spatially restrict proteins to their site of function and thus improve the probability that they will interact with their proper partners. In particular, the targeting of proteins to specific membrane-bound organelles has proven to be an effective cellular mechanism in maintaining the fidelity and efficiency of protein activities. Research within the past decade has identified protein domains that specifically bind the phosphatidylinositol (PtdIns) phospholipids, collectively called phosphoinositides (PIs), as major determinants in localizing proteins to their site of function (*1, 2*). These PI-binding motifs, which include the C2 (PKC conserved region 2), PH (Pleckstrin homology), FYVE (Fab1p/YOTP/Vac1p/EEA1), ENTH (Epsin NH_2-terminal homology) and tubby domains, are found in proteins implicated in a diverse array of cellular processes, such as protein transport, exocytosis, endocytosis, actin cytoskeletal organization, cell growth regulation, and control of gene expression. Through the regulated synthesis of distinct PIs on specific organelles, proteins containing these lipid-binding domains can be targeted and activated at the appropriate site of function. The importance of membrane targeting by PIs is exemplified by a number of human diseases linked to defects in PI signaling (*3–5*), including cancer, immunodeficiency disorders (X-linked agammaglobulinemina and chronic granulomatous disease), myotubular myopathy, kidney and neurological diseases (oculocerebro-renal syndrome of Lowe), and faciogenital dysplasia (Aarskog-Scott syndrome).

Even with the large number of PI-binding proteins previously identified, genetic and biochemical studies suggest the existence of additional effector molecules. For example, it has long been known that PI synthesis is necessary for the generation of superoxides by the human NADPH oxidase complex, though the connection between these processes had been elusive. Recently, it was determined that Phox Homology (PX) domains, including those in two NADPH oxidase subunits, bind to PIs, identifying another family of effector proteins [(*6–11*); reviewed in (*12*)]. Many members of this effector family contain additional motifs that mediate protein-protein interactions and other biochemical activities, such as protein phosphorylation and lipid modification (*13*). As with other lipid-binding motifs, PX domains play important roles in ensuring that proteins reach their appropriate intracellular location through the binding of membrane-restricted PIs.

PI Lipids and PI Kinases

In contrast to the headgroups of other phospholipids, the biological versatility of PtdIns is derived from its unique ability to be reversibly phosphorylated at three distinct positions of the inositol headgroup (Fig. 1). Single or combinatorial phosphorylation of the D-3, D-4, and D-5 positions on the inositol ring of PtdIns can generate at least seven unique PI derivatives. Furthermore, linkage between the inositol ring to diacylglycerol anchors PIs within lipid membranes. Thus, simple changes in the phosphorylation of PtdIns can trigger a number of distinct, membrane-restricted signals.

The biological activity of PIs can be

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