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Barcoding Hedgehog for Intracellular Transport

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Abstract

Hedgehog, an essential protein for the development of many vertebrate and invertebrate organs, signals at both short and long distances to control growth and patterning. The mechanism by which it moves between source and target cells is not known, but characterization of the covalent modification of the N-terminus with palmitate and of its C-terminus with cholesterol has led to the suggestion that the lipophilic properties of the modified protein serve to regulate movement after its secretion into the extracellular space. Another interpretation and model is that the C-terminal cholesterol acts to target Hedgehog to an intracellular trafficking pathway that prepares Hedgehog for release in an encapsulated form.

There are more than thirty known covalent modifications of proteins. Some modifications are additive and attach functional groups such as acetate, phosphate, phosphatidylinositol, sulfate, palmitate, polysialic acid, iodine, nucleotide, lipid or carbohydrate; prosthetic groups, such as heme; or entire proteins, such as ubiquitin, SUMO, Nedd or biotin. Some modifications are subtractive and remove single or multiple residues from the N- or C-terminal ends. Some modifications are structural in that they alter amino acids by changing the nature or form of a residue (for example, citrullination, crosslinking of cysteine residues with disulfides or proline racemization). Some modifications are permanent and are essential for secondary structure, tertiary structure, or activity; others are reversible and regulate activity. Some modifications determine stability, and others dictate location. This essay examines the cholesterol modification of the Hedgehog (Hh) protein and explores the possibility that cholesterol is an intracellular address label that directs Hh to a trafficking pathway in both the cells that produce Hh as well as the cells that receive it.

The Hh field emerged from studies of segmentation of the *Drosophila* embryo, where *hh* was first recognized as a gene that is needed to organize segments and maintain segment boundaries (1). Hh is now known to regulate growth and patterning of most tissues and organs in both invertebrates and vertebrates and to be involved in perturbations that lead to various human diseases. It is one of the central regulators of animal development.

In addition to its importance to development and disease, the Hh protein has attracted interest due to its unusual posttranslational processing (Fig. 1). A series of pioneering studies principally by the Beachy group showed that biogenesis of *Drosophila* Hh protein involves removal of its unusually long signal sequence, followed by autoproteolytic

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cleavage to generate an N-terminal 174 residue fragment (Hh-N) that is covalently linked to cholesterol at its C-terminus (2) and to palmitate at its N-terminus (p Hh-N_C) (3–5). Due to a combination of factors – including perhaps the importance of cholesterol to cardiovascular disease, the enigmatic physiological role of cholesterol, and the rarity of cholesterol as a protein modification – many studies have been carried out with the goal of understanding the role of Hh's cholesterol tail. Hh is a paracrine signal that targets cells at both short and long distances, and studies of its biogenesis have indicated that potency and ability to signal at a distance are affected if Hh is not modified with cholesterol. The widely accepted model for Hh function posits that proteolysis is an obligate step for activation of the Hh zymogen and that the cholesterol tail regulates Hh secretion and dispersion. I re-examine these accepted tenets of Hh signaling in light of several unexpected findings.

Autoproteolysis splits uncleaved Hh into two parts, generating modified p Hh-N_C and unmodified Hh C-terminus (Hh-C), and every assessment of Hh functionality has localized signaling activity to the Hh-N domain. For example, phenotypes caused by ectopic expression of wild-type Hh can be mimicked by expression of a mutant p Hh-N form that lacks a cholesterol tail, but not of Hh-C (6). (Because the Hh-N forms are active and do not compete with wild-type protein, whereas protein that lacks a palmitate N-terminal cap has dominant negative activity (7), it seems reasonable to assume that the Hh-N truncation mutants are acylated and should be designated p Hh-N.) Because missense mutations in Hh-N that cause *hh* loss-of-function do not affect autoproteolysis, but mutations that eliminate proteolysis map to Hh-C (8), the conclusion that Hh signaling functionality resides in the N-terminal 174 residues is well-supported.

The Hh-C domain provides at least two functions. It contains the protease site (8), and is responsible for axonal targeting (9) in retinal axons where Hh signals from axon termini to induce development of postsynaptic neurons in the brain (10). However, these findings do not indicate whether the uncleaved protein has activity. Indeed, analysis of the Hh His³²⁹→Ala (Hh^{H329A}) mutant protein suggests that uncleaved Hh is active. The engineering of this mutant was based on the shared catalytic mechanism of serine proteases in which a His residue in the active site acts as a general base, on the relative position of His³²⁹ in Hh-C, and on the evolutionary conservation of this His residue in Hh sequences; His³²⁹ has therefore been proposed to be part of the catalytic triad in the protease site of Hh (6). Hh^{H329A} is inactive as a protease (as measured by Western assays for processed N-terminal Hh), but its potency (as measured by its induction of gene expression in embryos) is reduced by only an estimated three-fold relative to wild type (6).

Another protease-deficient Hh mutant protein, Hh Cys²⁵⁸→Ala (Hh^{C258A}), in which Ala substitutes for Cys at the cleavage site, did not show activity in ectopic expression tests (8). It is not clear how to reconcile these apparently contradictory results - whether the partially active Hh^{H329A} protein retains basal autoproteolytic function and produces some p Hh-N_C, or whether the assays of Hh^{C258A} activity were not sufficiently sensitive. I note that both loss- and gain-of-function *hh* mutant phenotypes are variable and that the tests of Hh^{C258A} activity that yielded negative results involved time-limited, heat shock-induced expression. Moreover, findings from the Robbins laboratory show that various forms of both *Drosophila* Hh and vertebrate Shh proteins are active as uncleaved proteins (11). Tokhunts *et al.* (2009)

showed that p^{Hh}-U is active in assays in cultured vertebrate cells and can partially rescue *hh* phenotypes when ectopically expressed in fly eye imaginal discs. Although it remains uncertain whether p^{Hh}-U is active as a paracrine signaling protein under normal conditions, the activities of p^{Hh}-U and p^{Hh}-N, neither of which has the C-terminal cholesterol modification, have interesting implications for the role of Hh's cholesterol tail.

p^{Hh}-N can be engineered to terminate at Gly²⁵⁷, the site of normal autoproteolytic cleavage, and both *Drosophila* and vertebrate versions (p^{Hh}-N and p^{Shh}-N, respectively) have been extensively characterized in cell culture and in vivo assays. Every study reported to date has shown that lack of cholesterol modification affects signaling activity (7, 11–16). Models put forward to explain the functionality of p^{Hh}-N have focused on two aspects of Hh behavior. First, p^{Hh}-N_C associates tightly with the plasma membrane of producing cells, a property that is consistent with a role for cholesterol in anchoring proteins to membranes (17). Second, Hh protein multimerizes in solution (7, 18–21). Most models that address the function of Hh's cholesterol tail have focused on a role promoting membrane tethering, which would dictate how efficiently Hh is released from producing cells or adheres to target cells. Others have also suggested a role in the formation of multimers whose unique properties would presumably impact long-range movement. I suggest an alternative.

The Guerrero laboratory discovered a complex choreography that prepares Hh for release and dispersion (Fig. 2A). Newly synthesized Hh localized to the apical surface of wing disc epithelial cells, but was not released. Rather, after it was externalized apically, it was endocytosed in a dynamin-dependent process that sequestered it in vesicles that move through the cell to the basolateral compartment. These Hh-containing vesicles then associated with and traveled in a directional fashion along basal cytonemes to be delivered to target cells at the point of contact (22).

Although our understanding of intracellular protein trafficking is too rudimentary to explain why or how Hh is shuttled between apical and basolateral compartments, apical membranes have cholesterol and glycosphingolipid microdomains to which a class of proteins sort (23). Early studies described Hh distribution as basolateral and punctate (6, 24, 25), consistent with the findings of Callejo *et al.* (2011). In contrast, the truncation mutant p^{Hh}-N, which lacks cholesterol, was reported to have a diffuse, apical distribution (16). Perhaps cholesterol modification engages Hh in the pathway of apical membrane association, dynamin-dependent endocytosis and encapsulation in vesicles, but mutant protein that lacks cholesterol (for example, p^{Hh}-N and p^{Hh}-U) is not appropriately targeted. In this model, p^{Hh}-N and p^{Hh}-U cannot participate in the process that normally delivers p^{Hh}-N_C to target cells through the cytoneme-mediated basal route (Fig. 2B). Instead, for example, unregulated release of the non-cholesterol-modified p^{Hh}-N could occur by abnormal mechanisms as unusually high concentrations build up apically. Accordingly, the long-distance signaling produced by p^{Hh}-N may be greater (7, 12, 13, 16) or reduced (11, 14, 15) compared to p^{Hh}-N_C depending on the amount of protein that is released and the sensitivity of the cells that respond to it.

It can be difficult to infer wild-type function from mutant phenotypes, and for a complex process such as the one that orchestrates modification, trafficking and movement of Hh,

blocking a step early in the pathway could have manifold consequences. Thus, the altered range of p^{Hh}-N should be understood as an indirect consequence of lack of engagement in the normal process for release, and should not be taken as an indication that the role of cholesterol is to directly limit range of Hh movement. Once a signaling protein is released by a method other than cytoneme-based delivery, it cannot be controlled and its dispersion will be a function of accessibility of receptors and the nature of the extra-cellular milieu. The key point is that Hh lacking the cholesterol modification accumulates apically, suggesting that the cholesterol is a tag that targets Hh to a particular intracellular compartment, and that it is part of a protein barcode for an intracellular positioning system that can facilitate recruitment of Hh to an endocytic pathway for basal export. Re-localization of cholesterol-modified Hh to the basolateral compartment does not eliminate the possibility that Hh can also be exported apically, but the function of this barcode is apparently required for basal release. It could presumably function in both the producing cell in which cholesterol is needed for capture by dynamin-dependent endocytosis, and in the receiving cell where it might direct Hh to the site where the relevant components of signal transduction are localized.

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Fig. 1.
Wild-type and variant forms of Hedgehog.

Fig. 2.

Model of Hh transit in a Hh-producing cell. **(A)** Hh is synthesized and transported to the apical membrane (left panel). Hh that is modified by cholesterol can be captured by endocytosis into vesicles that mature and move to the basolateral membrane (middle panel). Hh-bearing vesicles are exported through cytonemes at the basolateral membrane (right panel). **(B)** Model of movement of non-cholesterol-modified Hh-N to the apical

compartment (left panel), accumulation apically and on the apical membrane (middle panel), and eventually to apical release (right panel).