CELL BIOLOGY

Barcoding Hedgehog for Intracellular Transport

Thomas B. Kornberg*

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 its 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.

More than 30 covalent modifications of proteins are known. 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, cross-linking 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 Perspective 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 and 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.

Cardiovascular Research Institute, University of California, San Francisco, CA 94158, USA.

***Corresponding author. E-mail, tkornberg@ucsf.edu**

In addition to its importance to development and disease, the Hh protein has attracted interest because of 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 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

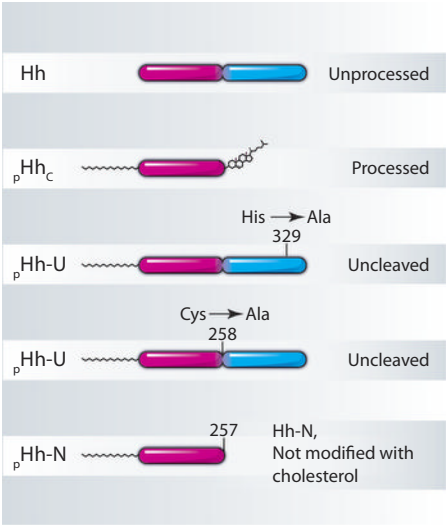

Fig. 1. Wild-type and variant forms of Hedgehog.

at its N terminus ($_p$Hh-N_c) (*3*–*5*). Because of a combination of factors—including the importance of cholesterol in 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 reexamine 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 by expression 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, whereas 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 active site (*8*), and it 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 $His^{329} \rightarrow Ala$ (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 His^{329} in Hh-C, and on the evolutionary conservation of this His residue in Hh sequences; His^{329} 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 a factor of ~3 relative to the wild type (*6*).

Another protease-deficient Hh mutant protein, Hh $Cys^{258} \rightarrow Ala$ (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 $_pHh\text{-}N_c$, or whether the assays of Hh^{C258A} activity were not sufficiently sensitive. Both loss-of-function and gain-of-function *hh* mutant phenotypes are variable, and 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; Tokhunts *et al.* (*11*) showed that $_pHh\text{-}U$ 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 $_pHh\text{-}U$ is active as a paracrine signaling protein under normal conditions, the activities of $_pHh\text{-}U$ and $_pHh\text{-}N$—neither of which has the C-terminal cholesterol modification—have interesting implications for the role of Hh's cholesterol tail.

$_pHh\text{-}N$ can be engineered to terminate at Gly^{257}, the site of normal autoproteolytic cleavage, and both *Drosophila* and vertebrate versions ($_pHh\text{-}N$ and $_pShh\text{-}N$, 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 $_pHh\text{-}N$ have focused on two aspects of Hh behavior. First, $_pHh\text{-}N_c$ associates tightly with the plasma membrane of Hh-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 in 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 affect long-range movement. I suggest an alternative.

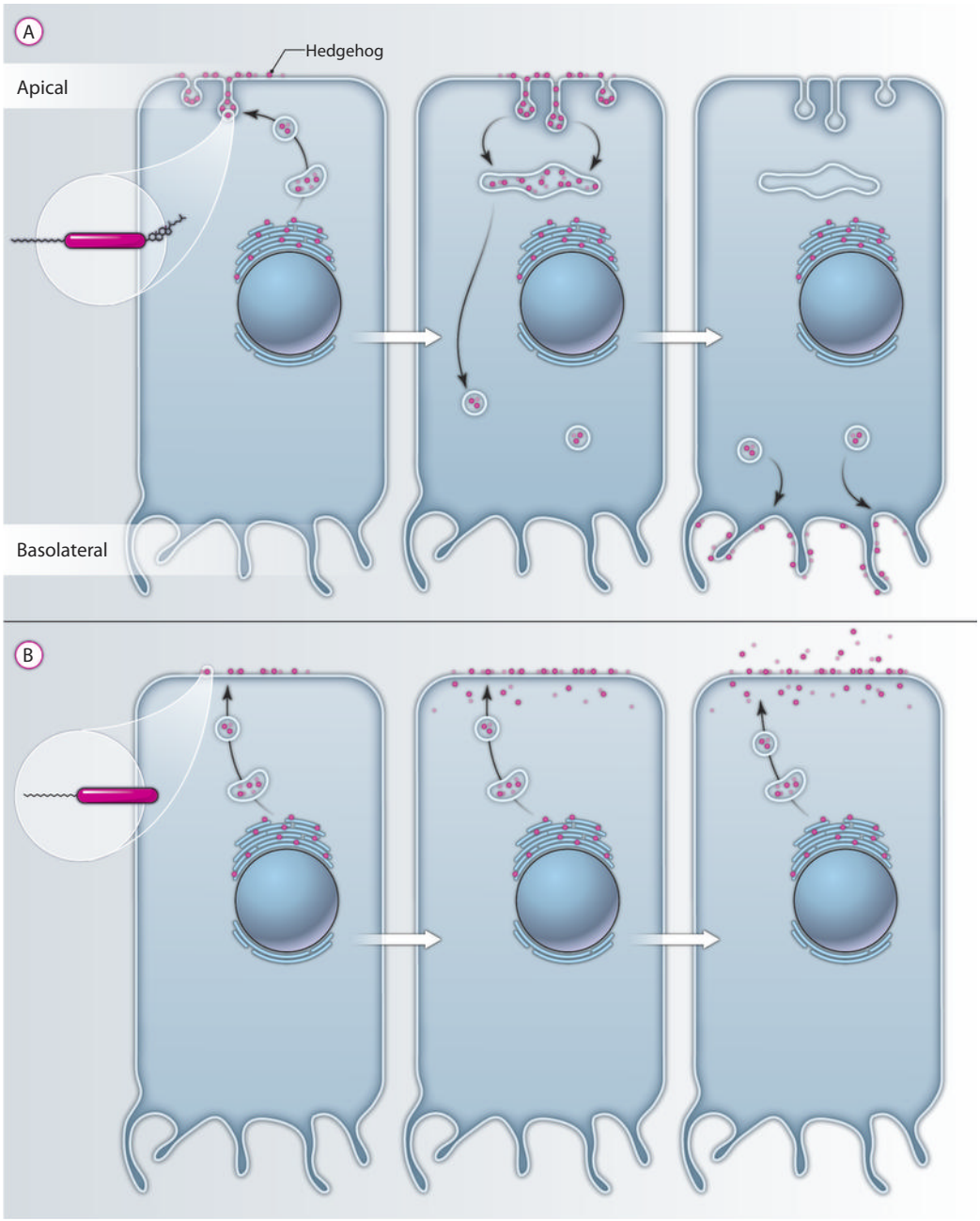

Fig. 2. Model of Hh transit in a Hh-producing cell. (**A**) Hh is synthesized and transported to the apical membrane (left panel). Hh that is modified by cholesterol can be captured by endocytosis into vesicles that mature and move to the basolateral membrane (center panel). Hh-bearing vesicles are exported 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).

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 moved 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*. (*22*). 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, whereas 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*) relative 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 the 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 extracellular milieu. The key point is that Hh lacking the cholesterol modification accumulates apically, which implies that cholesterol is a tag that targets Hh to a particular intracellular compartment, and that the cholesterol tag is part of a protein barcode for an intracellular positioning system that can facilitate recruitment of Hh to an endocytic pathway for basal export. Relocalization 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 Hh-producing cell in which cholesterol is needed for capture by dynamin-dependent endocytosis, and in the Hh-receiving cell where it might direct Hh to the site where the relevant components of signal transduction are localized.

References

1. C. Nüsslein-Volhard, E. Wieschaus, Mutations affecting segment number and polarity in *Drosophila*. *Nature* **287**, 795–801 (1980).
2. J. A. Porter, K. E. Young, P. A. Beachy, Cholesterol modification of hedgehog signaling proteins in animal development. *Science* **274**, 255–259 (1996).
3. K. Amanai, J. Jiang, Distinct roles of Central missing and Dispatched in sending the Hedgehog signal. *Development* **128**, 5119–5127 (2001).
4. J. D. Lee, J. E. Treisman, Sightless has homology to transmembrane acyltransferases and is required to generate active Hedgehog protein. *Curr. Biol.* **11**, 1147–1152 (2001).
5. C. A. Micchelli, I. The, E. Selva, V. Mogila, N. Perrimon, Rasp, a putative transmembrane acyltransferase, is required for Hedgehog signaling. *Development* **129**, 843–851 (2002).
6. J. J. Lee, S. C. Ekker, D. P. von Kessler, J. A. Porter, B. I. Sun, P. A. Beachy, Autoproteolysis in hedgehog protein biogenesis. *Science* **266**, 1528–1537 (1994).
7. A. Gallet, L. Ruel, L. Staccini-Lavenant, P. P. Thérond, Cholesterol modification is necessary for controlled planar long-range activity of Hedgehog in *Drosophila* epithelia. *Development* **133**, 407–418 (2006).
8. J. A. Porter, D. P. von Kessler, S. C. Ekker, K. E. Young, J. J. Lee, K. Moses, P. A. Beachy, The product of hedgehog autoproteolytic cleavage active in local and long-range signalling. *Nature* **374**, 363–366 (1995).
9. T. Chu, M. Chiu, E. Zhang, S. Kunes, A C-terminal motif targets Hedgehog to axons, coordinating assembly of the *Drosophila* eye and brain. *Dev. Cell* **10**, 635–646 (2006).
10. Z. Huang, S. Kunes, Hedgehog, transmitted along retinal axons, triggers neurogenesis in the developing visual centers of the *Drosophila* brain. *Cell* **86**, 411–422 (1996).
11. R. Tokhunts, S. Singh, T. Chu, G. D'Angelo, V. Baubet, J. A. Goetz, Z. Huang, Z. Yuan, M. Ascano, Y. Zavros, P. P. Thérond, S. Kunes, N. Dahmane, D. J. Robbins, The full-length unprocessed hedgehog protein is an active signaling molecule. *J. Biol. Chem.* **285**, 2562–2568 (2010).
12. A. Callejo, C. Torroja, L. Quijada, I. Guerrero, Hedgehog lipid modifications are required for Hedgehog stabilization in the extracellular matrix. *Development* **133**, 471–483 (2006).
13. R. J. Dawber, S. Hebbes, B. Herpers, F. Docquier, M. van den Heuvel, Differential range and activity of various forms of the Hedgehog protein. *BMC Dev. Biol.* **5**, 21 (2005).
14. A. Gallet, R. Rodriguez, L. Ruel, P. P. Therond, Cholesterol modification of hedgehog is required for trafficking and movement, revealing an asymmetric cellular response to hedgehog. *Dev. Cell* **4**, 191–204 (2003).
15. P. M. Lewis, M. P. Dunn, J. A. McMahon, M. Logan, J. F. Martin, B. St-Jacques, A. P. McMahon, Cholesterol modification of sonic hedgehog is required for long-range signaling activity and effective modulation of signaling by Ptc1. *Cell* **105**, 599–612 (2001).
16. J. A. Porter, S. C. Ekker, W. J. Park, D. P. von Kessler, K. E. Young, C. H. Chen, Y. Ma, A. S. Woods, R. J. Cotter, E. V. Koonin, P. A. Beachy, Hedgehog patterning activity: role of a lipophilic modification mediated by the carboxy-terminal autoprocessing domain. *Cell* **86**, 21–34 (1996).
17. C. Peters, A. Wolf, M. Wagner, J. Kuhlmann, H. Waldmann, The cholesterol membrane anchor of the Hedgehog protein confers stable membrane association to lipid-modified proteins. *Proc. Natl. Acad. Sci. U.S.A.* **101**, 8531–8536 (2004).
18. M. H. Chen, Y. J. Li, T. Kawakami, S. M. Xu, P. T. Chuang, Palmitoylation is required for the production of a soluble multimeric Hedgehog protein complex and long-range signaling in vertebrates. *Genes Dev.* **18**, 641–659 (2004).
19. J. Feng, B. White, O. V. Tyurina, B. Guner, T. Larson, H. Y. Lee, R. O. Karlstrom, J. D. Kohtz, Synergistic and antagonistic roles of the Sonic hedgehog N- and C-terminal lipids. *Development* **131**, 4357–4370 (2004).
20. J. A. Goetz, S. Singh, L. M. Suber, F. J. Kull, D. J. Robbins, A highly conserved amino-terminal region of sonic hedgehog is required for the formation of its freely diffusible multimeric form. *J. Biol. Chem.* **281**, 4087–4093 (2006).
21. X. Zeng, J. A. Goetz, L. M. Suber, W. J. Scott, Jr, C. M. Schreiner, D. J. Robbins, A freely diffusible form of Sonic hedgehog mediates long-range signalling. *Nature* **411**, 716–720 (2001).
22. A. Callejo, A. Bilioni, E. Mollica, N. Gorfinkiel, G. Andrés, C. Ibáñez, C. Torroja, L. Doglio, J. Sierra, I. Guerrero, Dispatched mediates Hedgehog basolateral release to form the long-range morphogenetic gradient in the *Drosophila* wing disk epithelium. *Proc. Natl. Acad. Sci. U.S.A.* **108**, 12591–12598 (2011).
23. K. Simons, E. Ikonen, Functional rafts in cell membranes. *Nature* **387**, 569–572 (1997).
24. T. Tabata, T. B. Kornberg, Hedgehog is a signaling protein with a key role in patterning *Drosophila* imaginal discs. *Cell* **76**, 89–102 (1994).
25. A. M. Taylor, Y. Nakano, J. Mohler, P. W. Ingham, Contrasting distributions of patched and hedgehog proteins in the *Drosophila* embryo. *Mech. Dev.* **42**, 89–96 (1993).

10.1126/scisignal.2002447

Citation: T. B. Kornberg, Barcoding Hedgehog for intracellular transport. *Sci. Signal.* **4**, pe44 (2011).