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NOVEL LIPID MODIFICATIONS OF SECRETED PROTEIN SIGNALS

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■ **Abstract** Secreted signaling proteins function in a diverse array of essential patterning events during metazoan development, ranging from embryonic segmentation in insects to neural tube differentiation in vertebrates. These proteins generally are expressed in a localized manner, and they may elicit distinct concentration-dependent responses in the cells of surrounding tissues and structures, thus functioning as morphogens that specify the pattern of cellular responses by their tissue distribution. Given the importance of signal distribution, it is notable that the Hedgehog (Hh) and Wnt proteins, two of the most important families of such signals, are known to be covalently modified by lipid moieties, the membrane-anchoring properties of which are not consistent with passive models of protein mobilization within tissues. This review focuses on the mechanisms underlying biogenesis of the mature Hh proteins, which are dually modified by cholesteryl and palmitoyl adducts, as well as on the relationship between Hh proteins and the self-splicing proteins (i.e., proteins containing inteins) and the Hh-like proteins of nematodes. We further discuss the cellular mechanisms that have evolved to handle lipidated Hh proteins in the spatial deployment of the signal in developing tissues and the more recent findings that implicate palmitate modification as an important feature of Wnt signaling proteins.

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INTRODUCTION

Pattern formation during embryonic development is directed by several families of secreted protein signals. Whereas some of these signals are limited to local action at emerging tissue interfaces, others exert their influence over a range of distances. The patterning effects of these signaling proteins result from their action as morphogens, that is, by eliciting concentration-dependent responses in cells surrounding a localized site of signal production and release. The patterns of cell proliferation and differentiation resulting from the action of such a morphogen depend critically upon the response of target cells within a tissue and, of course, upon the tissue distribution of the active signal itself. We focus on lipid modification as a factor that critically influences the distribution and activity of Hedgehog (Hh) and Wnt signaling proteins, which act as morphogens in specifying the patterns of proliferation and differentiation in many tissues and structures during embryogenesis.

First discovered in *Drosophila*, the Hh family of secreted signaling proteins has been studied in a wide array of metazoan organisms. The cellular machinery that generates, distributes, transduces, and ultimately implements a cellular response to Hh signals is deployed repeatedly throughout development, and this pathway directly or indirectly influences the development of many tissue and organ systems in vertebrates [for recent reviews, see (1, 2)]. Wnt proteins similarly act in the embryonic patterning of many tissues and structures [see (3–5)]. More recently, the Hh and Wnt signaling pathways are emerging as playing homeostatic roles in the maintenance of postembryonic tissues. Such roles include stem cell maintenance in particular tissues (6–10) and possible participation in tissue repair in response to injury (11). Finally, both the Hh and Wnt pathways have emerged as playing an important role in a growing number of types of cancer (11–13) in which a general feature is unregulated activation of these pathways [see (14–16)].

The normal and abnormal function of these pathways is associated with diverse biological phenomena of considerable importance and interest. Our discussion, however, will focus on the distribution and activity of these signaling proteins, which are critical determinants of their biological effects, and in particular on the mechanisms by which these proteins are lipid modified and how these modifications impact their biological function. Well-known mechanisms

that influence the distribution and activity of extracellular protein signals include interactions with the cell matrix (e.g., FGF) (17), release in latent form (e.g., TGFβ) (18), or interaction with secreted inhibitors (e.g., Wnts and BMPs) (19, 20). The lipid modifications undergone by Hh proteins generally are considered to be membrane anchoring, raising the question as to how signaling responses in distant cells are elicited. We discuss the following:

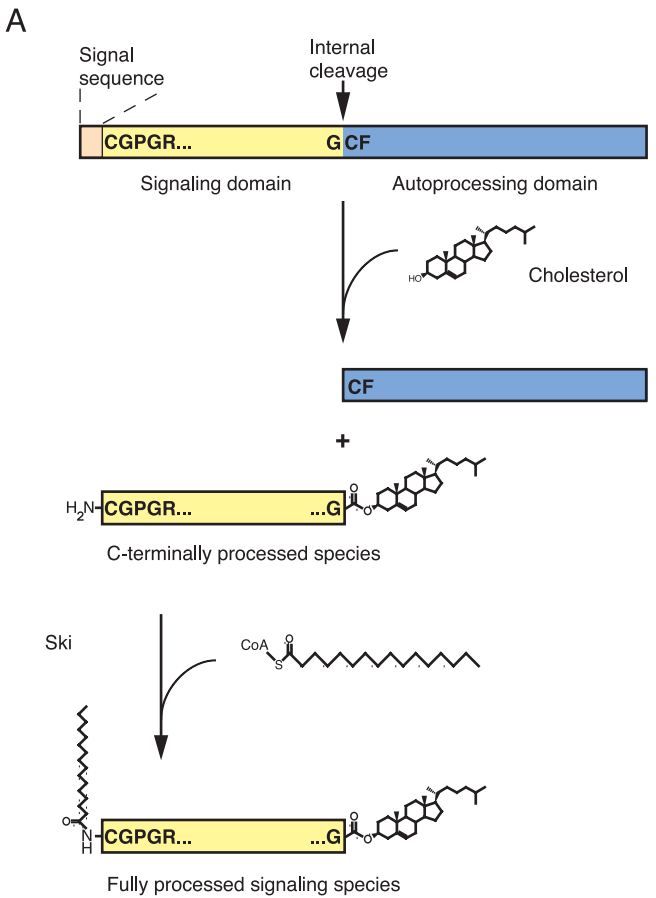
- the biochemical mechanisms of these modifications, which include autoprocessing and cholesterol modification of the Hh signal, and further acylation of the Hh protein;
- the more recent finding of Wnt protein acylation, which may occur by a mechanism related to that of Hh protein acylation;
- the relationship between Hh and self-splicing proteins, a well-studied class of autoprocessing proteins, as well as the Hh-like proteins of nematodes; and
- finally, the dual lipid modification of Hh proteins in the context of lipid cell biology and metabolism and, largely in the context of *Drosophila* development, how these hydrophobic modifications target Hh protein to a multicomponent system for distribution of the signaling activity in its appropriate pattern within tissues.

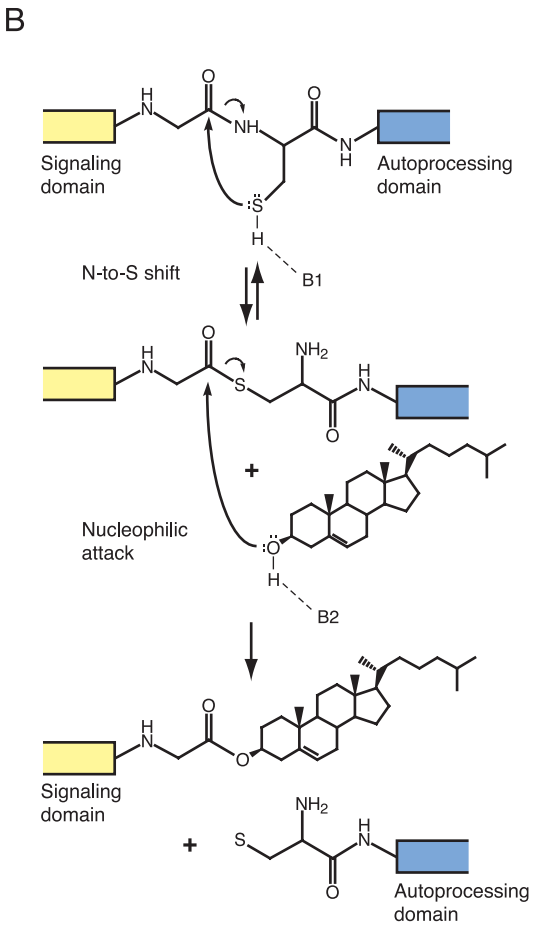
BIOGENESIS OF HEDGEHOG SIGNALING PROTEINS

Newly synthesized Hh proteins undergo a series of posttranslational processing reactions within the secretory pathway that result in the formation and cell surface presentation of the species active in signaling. Although elements of the reaction mechanisms employed are also represented in the metabolism of other proteins, Hedgehog family members are the only examples of signaling proteins known to be covalently modified by cholesterol. In this section, we describe the biogenesis of the Hedgehog signal as compared to that of other autoprocessed proteins. In addition, we review the biochemistry and function of Hedgehog palmitoylation.

Autocatalytic Processing and Cholesterol Modification of Hedgehog Proteins

Whereas many features of Hh autoprocessing [reviewed in (21)] were elucidated from studies of the *Drosophila* protein, the biochemical and functional mechanisms likely apply to Hh proteins from all species. Following cleavage of an amino-terminal signal sequence upon entry into the secretory pathway, the Hh protein undergoes an autocatalytic processing reaction that involves internal cleavage between Gly-Cys residues that form part of an absolutely conserved Gly-Cys-Phe tripeptide (Figure 1*A*) (22, 23). The amino-terminal product of this





cleavage receives a covalent cholesteryl adduct (24) and is the species active in signaling (23, 25–31) (see Figure 1*A*). Constructs encoding Hh proteins truncated at the normal site of internal cleavage produce proteins that can have signaling activity, but studies in *Drosophila* have demonstrated that such proteins are not appropriately restricted spatially and therefore cause gross mispatterning and lethality in embryos (32). The cholesteryl moiety not only restricts spatial deployment of the mature signal via insertion into the lipid bilayer of the cell membrane, thus influencing the pattern of cellular responses in developing tissues, but it may also function as an essential molecular handle for proper intracellular and extracellular trafficking and localization of the signal (see below). The autoprocessing reaction thus is required not only to release the active signal from precursor but also to specify the properties of this signal within cells and tissues.

The autoprocessing reaction is mediated by the carboxy-terminal domain of the Hh precursor, which has no known additional function. This reaction proceeds by two sequential nucleophilic displacements (Figure 1*B*): The first of which is a rearrangement to replace the main chain peptide linkage between Gly-Cys with a thioester involving the Cys side chain (24, 32). The second step of the Hh autoprocessing reaction involves attack upon the same carbonyl by a second nucleophile, displacing the sulfur and severing the link between Hh-N and Hh-C. The requirement for a second nucleophile in vitro can be met by a high concentration either of a thiol-containing molecule or of another small molecule with nucleophilic properties at neutral pH; these small nucleophiles can be shown to form covalent adducts to the amino-terminal product of the in vitro cleavage reaction (32). This second nucleophile can be provided by the thiol of a cysteine-initiated peptide, leading to a thioester linkage that undergoes further rearrangement via an S-to-N shift to form an amide (32). This reaction represents a variation on the theme of synthetic thioesters used for chemical ligation of peptides (33). Related protein ligation strategies are now used for synthesis of proteins containing specific modified or unnatural amino acids [see (34)].

The importance of autocatalytic processing in biogenesis of active Hh proteins is highlighted by the types of missense mutations that occur in the *Drosophila hh* gene (23). One class of mutations affects amino-terminal coding sequences without affecting the ability of the protein to undergo processing, and these

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Figure 1 Processing of Hedgehog proteins. (*A*) *Hedgehog* genes encode precursor polypeptides of ~45 kDa that undergo both N-terminal signal sequence trimming and acylation as well as internal proteolysis at a conserved sequence. Endoproteolytic cleavage at the GCF sequence is catalyzed by the processing activity associated with the C-terminal domain and produces an ~19 kDa segment with which all known signaling activities are associated. During cleavage, the signaling domain is modified at its carboxy-terminal glycine by cholesterol; the N-terminal cysteine also becomes palmitoylated (see text for details). (*B*) Mechanism of Hedgehog endoproteolysis (see text for details).

alterations affect either the secretion or the activity of the signaling domain. A second class of mutations comprises alterations of the carboxy-terminal domain without changing amino-terminal sequences, and these alterations can be shown to affect processing, thus demonstrating the requirement for processing in release of the active signal. Missense mutations in the human *Shh* gene associated with holoprosencephaly (see below) also can be classified in this manner, with alterations either in the amino-terminal signaling domain or the carboxy-terminal processing domain (35, 36).

Whereas cholesterol is a prominent constituent of animal cell membranes (as much as 30% of the plasma membrane lipid content in some tissues) and was identified as the modifying lipid in cell-derived Hh-Np (24), other steroidal compounds can substitute for cholesterol in Hh processing reactions performed in vitro (37). Among the compounds tested (see Figure 2), structural variables included the orientation and availability of the 3β hydroxyl, additional specific hydroxylations, the olefin of the cholestene backbone motif, as well as presence and structure of the isooctyl side chain. In this semiquantitative analysis, it is clear that the most important structural feature is the C3 hydroxyl moiety. Not only must the group be free of esterified adducts (no activity with cholesteryl acetate), but there is an absolute requirement for the β orientation of the alcohol (no activity with epicholesterol). Whereas the side chain of these sterols is not essential for activity (see 5-androsten-3β-ol), hydroxylations within the chain can decrease a compound's effectiveness (20-, 22-, 25-hydroxycholesterol). Hydroxylations at other positions within the ring can have no deleterious effect (7β-hydroxycholesterol) or can reduce activity (19-hydroxycholesterol). Finally, changing the cholestene to either a cholestan (coprostan-3-ol) or an ergostatrien (ergosterol) resulted in decreased activity, but this indicates that the olefin at C5 of the backbone is not essential and that conjugation does not prevent the esterification reaction.

The availability of alternative sterols, supplied nutritionally or biosynthetically, and the lack of stringent selectivity of the *Drosophila* Hh processing domain raise the possibility that hedgehog signaling proteins may be modified in vivo, not only by cholesterol, but by other endogenous steroidal nucleophiles. Although this remains to be demonstrated experimentally, it is worth noting that among Hh orthologs there is considerable sequence diversity within the subdomain that in *Drosophila* is known to be required for mediating cholesterol addition (see below).

Evidence for Additional Cholesterol-Modified Proteins

In animal cells, the processed form of overexpressed Sonic hedgehog signaling protein can be detected readily in simple metabolic labeling experiments using radioactive cholesterol (24). In addition to the strong signal due to incorporation within Shh-Np, however, several additional proteins can be detected. Although these proteins and the nature of the chemical linkage remain uncharacterized, this observation suggests that cholesterol modification of polypeptides, perhaps by

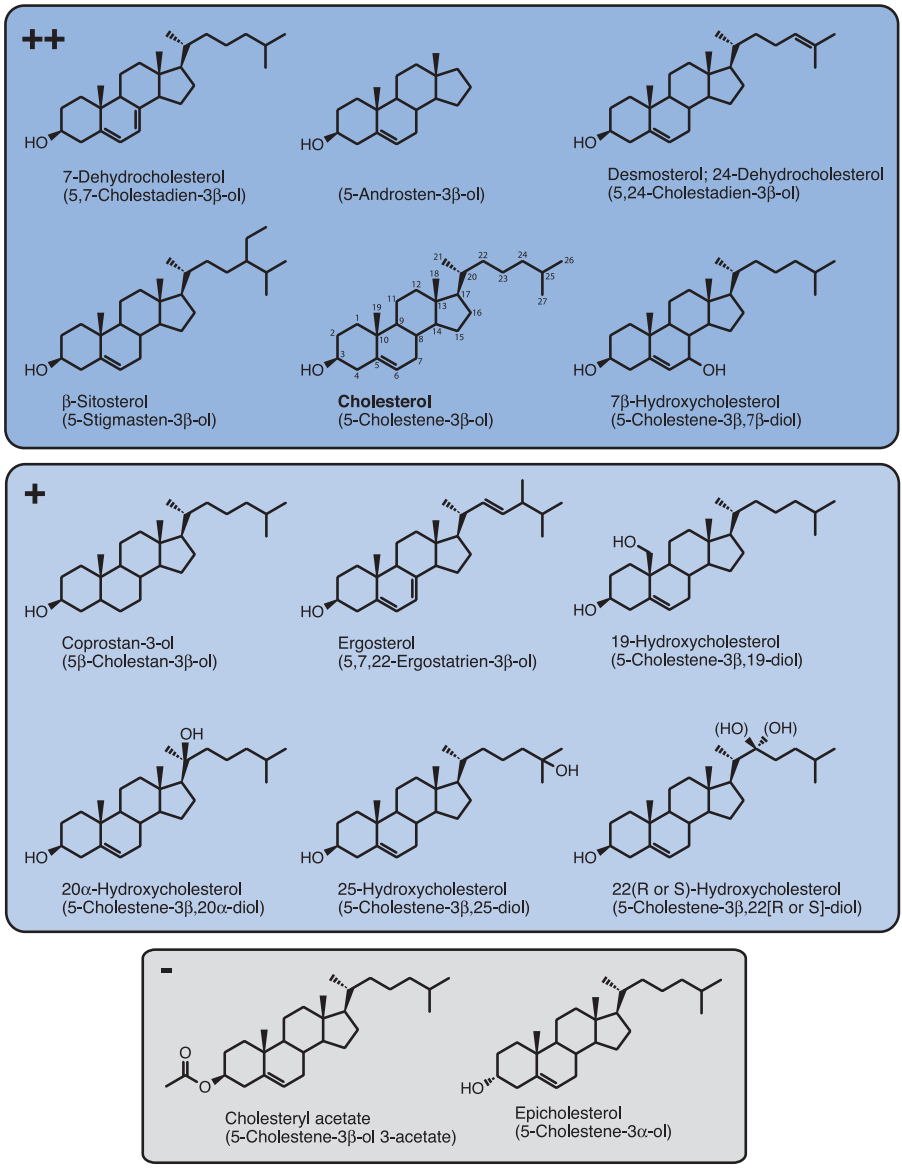


Figure 2 Sterol selectivity in the Hedgehog processing domain. Using a semiquantitative in vitro processing assay (37), the depicted sterols were tested for their ability to substitute for cholesterol in the processing domain-mediated transfer reaction. Full activity (++) is observed for compounds that most closely resemble cholesterol, with 5-androstene-3β-ol perhaps representing the minimum structural requirement for a fully active substrate. Various structural changes (see text) allow for partial activity (+), whereas esterification or inversion (to the α orientation) of the 3β hydroxyl completely block participation (-).

esterification, is not unique to the Hh proteins, and it may also be a employed as a means of directing other proteins to membranes or other hydrophobic targets. Alternatively, cholesterol labeling of these proteins could be caused by capture of oxidized sterol intermediates, as suggested for aldehyde dehydrogenase class 1 in bovine lens epithelial cells (38).

Thioesters as Intermediates in Protein Modification

The use of a Cys-derived thioester as an intermediate is a theme common to several other acyl transfers that result in covalent modifications of proteins [reviewed by (39) and (40)]. Following formation of the initial thioester in these systems, the acyl portion of the thioester (the acceptor, corresponding to Hh-N) can receive the final modification directly or alternatively may be transferred to other thiols in one or more subsequent steps before receiving the final modification. The ubiquitin cascade represents such a reaction with multiple intermediates, whose role is to attach ubiquitin to proteins destined for degradation by the proteasome (41). The acyl group for these thioesters is supplied by the carboxy-terminal Gly of ubiquitin, and the thiols come from Cys side chains in three distinct classes of enzymes. The first of these, E1, forms the initial thioester in an ATP-consuming reaction. Then, through transthioesterification reactions, the ubiquitin forms thioesters sequentially with E2 and E3 enzymes before final transfer to the ϵ amine of a Lys side chain. The protein receiving ubiquitin in the resulting amide linkage is thus marked for degradation.

The α2-macroglobulin proteinase inhibitors and the C3, C4, and C5 complement proteins represent members of an ancient superfamily that use an intrachain thioester as a "spring loaded" functionality, which can be triggered for covalent attachment to target molecules (42). The intrachain thioester is formed by thiol attack of a Cys side chain on the amido group of a Gln side chain. The final adducts in the case of the complement proteins are nucleophiles on the surface of cells to be targeted for lysis. In the α2-macroglobulin case, the final adduct is a nucleophile on a protease to be inactivated, which is targeted to α2-macroglobulin through the presence of multiple cleavage sites for proteases of various specificities.

In the examples just discussed, the acyl group contributing to the thioester intermediate derives either from another protein or from an amino acid side chain. In contrast, the acyl group in the Hh thioester intermediate is linked to a main chain carbonyl, and the thioester therefore replaces an amide bond within the peptide backbone. Other proteins likely to utilize main chain ester or thioester intermediates in autoprocessing reactions include prohistidine decarboxylase (43) and members of the Ntn hydrolase family that are processed by an intramolecular mechanism (44, 45). The Ntn (N-terminal nucleophile) family hydrolases, which include proteases active in the proteasome, are autoprocessed with internal cleavage, leaving the active site nucleophile as the amino-terminal residue. The role of these reactions appears to be activation of a precursor protein and takes place without net addition of a modifying adduct. There is no evidence

of any evolutionary relationship between Hh autoprocessing domains and either prohistidine decarboxylase or Ntn hydrolase proteins.

Ester Intermediates in Proteins Containing the Hint Domain

Two other groups of proteins that are evolutionarily related to Hh proteins are the self-splicing proteins and a group of novel nematode proteins containing Hh-C-like sequences. The self-splicing proteins undergo a reaction in which an internal portion of the protein, termed an intein, is excised and amino- and carboxy-terminal flanking regions, termed exteins, are ligated to form the mature protein (46, 47). Inteins are found inserted into a wide variety of archaeal, bacterial, chloroplast, and yeast proteins. The intein portion mediates the protein splicing reaction and typically also contains an endonuclease thought to act at the DNA level in mediating movement of intein coding sequences. Similar to Hh autoprocessing, the protein splicing reaction is initiated by intramolecular attack of a hydroxyl or thiol upon the preceding carbonyl, and the resulting ester or thioester intermediate replaces the peptide bond at the amino-terminal extein/intein boundary (48) (Figure 3). Unlike Hh proteins, the second nucleophilic attack in the protein self-splicing reaction involves the side chain of another Ser or Cys residue several hundred residues downstream. The resulting branched protein intermediate ultimately resolves into the ligated exteins and the free intein protein (Figure 3).

Nematode proteins with Hh-C-like sequences were identified by searching for homology within the *Caenorhabditis elegans* genomic sequence database. Within this genome, the sequencing of which is now complete (49), 10 putative proteins with homology to the Hh-C autoprocessing domain have been identified (32, 50–52; R. Mann, X. Wang, and P.A. Beachy, unpublished data). As in the Hh family, the Hh-C-like domains, with one exception, are located at the carboxy termini of these proteins and are preceded by an amino-terminal domain bearing a signal sequence. These nematode proteins resemble each other more than they do any other database sequence and can be grouped into three families based on their amino-terminal sequences: Wart, Ground, and a third family identified by the trivial name of its single member, M110. The structures of these proteins suggest the possibility that they are secreted and undergo autoprocessing; a preliminary study of one family member in *Drosophila* cultured cells indeed demonstrates cleavage at the junction between amino- and carboxy-terminal domains (24). Whereas there is no obvious similarity between the amino-terminal domains of the nematode and Hedgehog protein families, a short, shared sequence motif has been noted (52) that, in the context of cysteine conservation at flanking sites nearby, suggests they may share a common ancestor. In the absence of known structural conservation, however, the proposed evolutionary homology must be considered provisional.

The level of amino acid sequence identity between the nematode and Hh processing domains ranges from 24% to 32% in a region approximately corre-

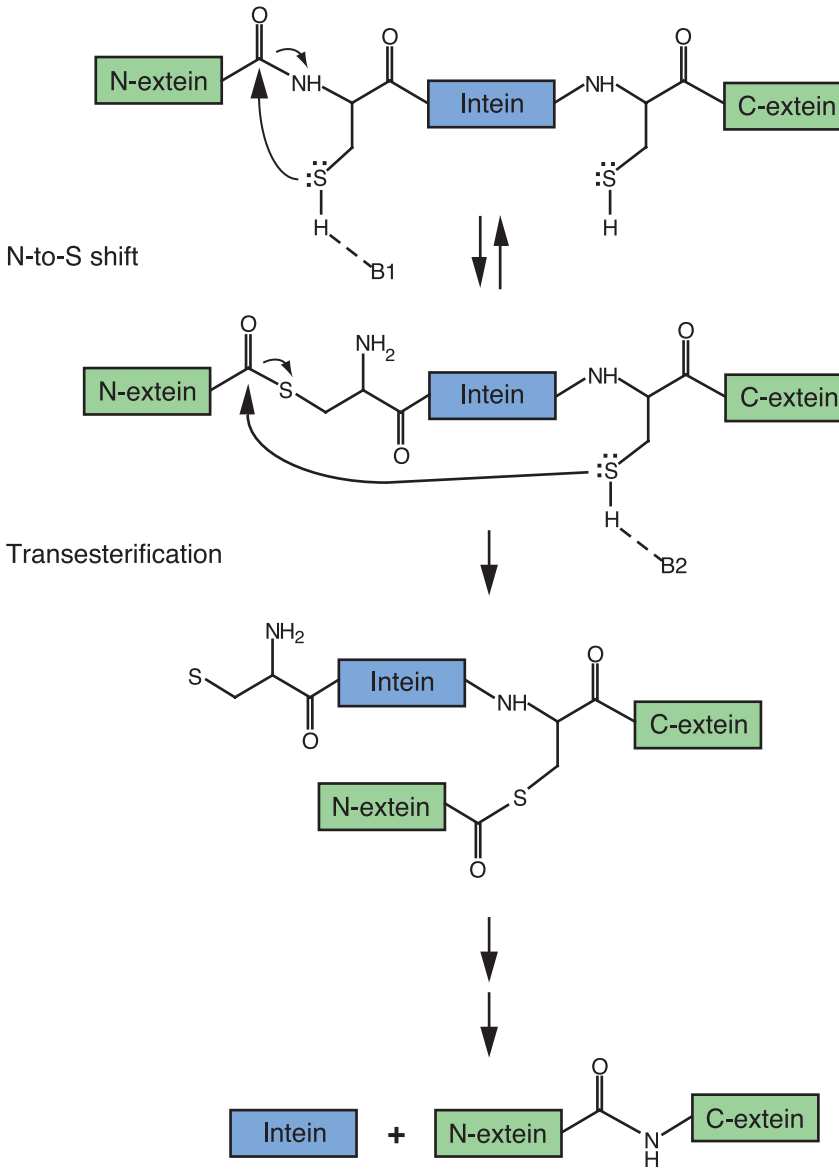


Figure 3 Mechanism of intein autoprocessing. The Hint domain-catalyzed N-to-S shift follows that of Hedgehog. However, the subsequent nucleophilic displacement is intramolecular, resulting in a branched polypeptide intermediate. This structure resolves into an excised intein sequence and a ligated amino- and carboxy-terminal segment [see (40, 46, 47) for recent reviews and reference to the intein database InBase].

sponding to the amino-terminal 2/3 of Hh-C. This same region of Hh-C also can be aligned with inteins, although the alignment is complicated by the presence of sequences corresponding to the endonuclease domain as well as a DNA recognition region domain that is thought to aid in DNA binding (51, 53, 54). The strongest evidence of a common evolutionary origin for these protein families is the presence of a domain with a common fold in the crystal structures of Hh-C and the 454 residue intein protein PI-SceI (Figure 4*A*) (51, 55). Remarkably, both the endonuclease (Endo) and DNA recognition region (DRR) domains are inserted into peripheral loops of the common domain with little apparent effect upon its three-dimensional fold. Structure-based alignment of Hh-C and PI-SceI intein sequences (Figure 4*B*; T. Hall and D. Leahy, personal communication), with intein endonuclease and DRR regions removed, reveals a low level of amino acid identity (15/94 residues aligned, ~16%), but the root-mean-square distance for Cα positions is 1.401 Å, and most of the residues known to be essential for Hh-C processing activity are conserved. The crystallized Hh fragment contains the 151 amino-terminal residues of Hh-C of which the first 145 residues are well ordered in the crystal structure; these residues correspond to the region conserved in the nematode proteins (9 identities among various Hh and the Wart/Ground proteins and most other positions featuring similar residues) [see (52)]. This domain alone suffices for thioester formation, as indicated by the ability of a Hh protein truncated after this point to undergo cleavage in the presence of DTT (51; X. Wang, G. Seydoux, and P.A. Beachy, unpublished information), and this domain has been referred to as the Hint module (Hedgehog, intein).

Although the Hint module in Hh-C suffices for the first step of autoprocessing, at least some part of the 63 carboxy-terminal residues missing in the crystallized fragment is required for the second step of cholesterol addition (51). Because of its apparent role in sterol addition, this 63 residue region is referred to as SRR, for sterol recognition region. Whereas residue identities between Hh SRRs and corresponding sequences within the nematode family are limited, the use of a short Hint sequence "anchor," as well as a gap to accommodate sequence insertions, particularly within the Shh and *Drosophila* proteins, reveals a significant degree of sequence similarity, most notably in the spacing of hydrophobic clusters (see Figure 5). Because of their corresponding position with respect to the SRR of Hh, the sequences in these nematode proteins that extend carboxy-terminal to the Hint domain are tentatively designated ARR, for adduct recognition region. Despite the above-mentioned similarities, the overall sequence diversity between the SRR of Hh proteins and the ARR regions of nematode gene family members raises the possibility that molecules other than cholesterol may participate in the processing reaction and form novel protein-modifying adducts.

From the structure of the Hint modules in Hh-C and the PI-SceI intein, and from sequence relationships between these proteins and the nematode proteins, a plausible evolutionary history can be constructed in which an ancestral Hint module evolved and gave rise to all three protein groups (see Figure 6). The evolution of the Hint module is revealed by pseudo twofold symmetry with

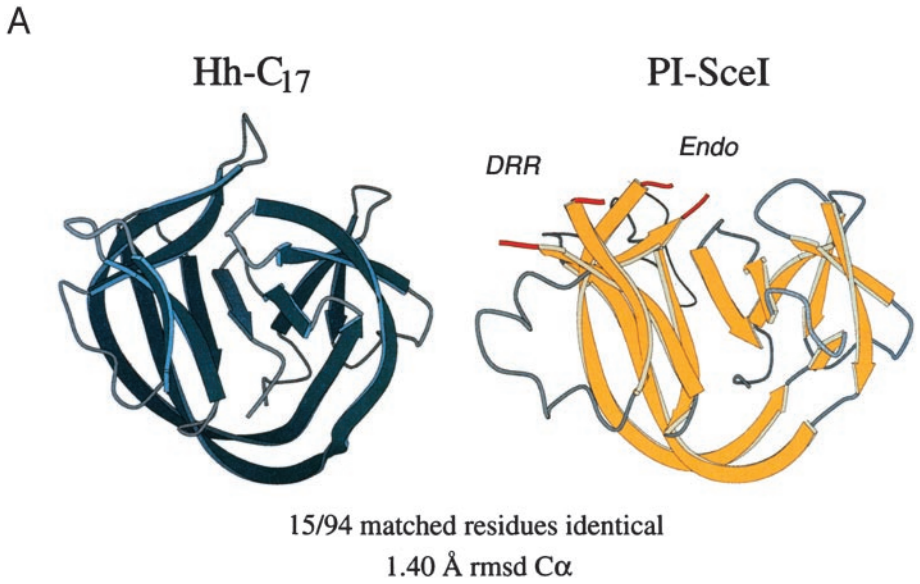


Figure 4 (*A*) Hint domain structures of Hh and an intein protein, PI-SceI. Crystal structures of both the Hedgehog processing domain (Hh-C_{17}) (51) as well as the PI-SceI autoprocessing protein (55) have been solved, and the Hint domain ribbon diagrams are presented for comparison. Structural similarities are easily detectable after removal of the DRR and endonuclease (Endo) domains of PI-SceI [see (51) for discussion of structural similarities as well as the interesting pseudo twofold axis of symmetry found in both structures]. (*B*) Structure-based alignment of Hint domain sequences from Hh and PI-SceI. The alignment of corresponding structures of the two Hint domains and their associated sequences allows a more accurate assessment of relatedness and reveals a greater degree of conservation. The analysis demonstrates that 94 amino acids are in matched positions, that ~16% of matched residues are identical, and that similar residues are found at most of the other positions. The root-mean-square distance for Cα positions is 1.401 Å.

superimposable subdomains in the crystal structure of the Hh-C protein, which suggests that the ancestral Hint domain arose by gene duplication (see Figure 4*A*) [see (51)]. The duplicated subdomains are interlinked by extended loop-like secondary structure elements that mirror each other in associating primarily with the bulk of the other subdomain, suggesting that following duplication, these secondary structure elements exchanged interactions with their own subdomain for similar interactions with the other subdomain [a "loop swap"; see (56) and Figure 6]. Following establishment of the Hint module, the ancestral intein evolved in one branch by insertion of an endonuclease into a Hint domain and by adjustment (or preservation) of the chemistry to insure that the second nucleophilic attack is made intramolecularly by the side chain of a downstream residue. In a second branch, Hh proteins were formed by association of a Hint domain

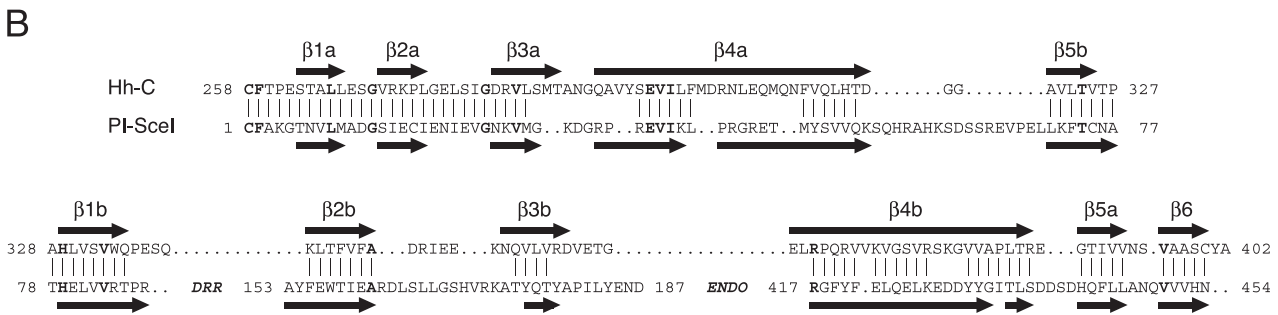


Figure 4 (*Continued*)

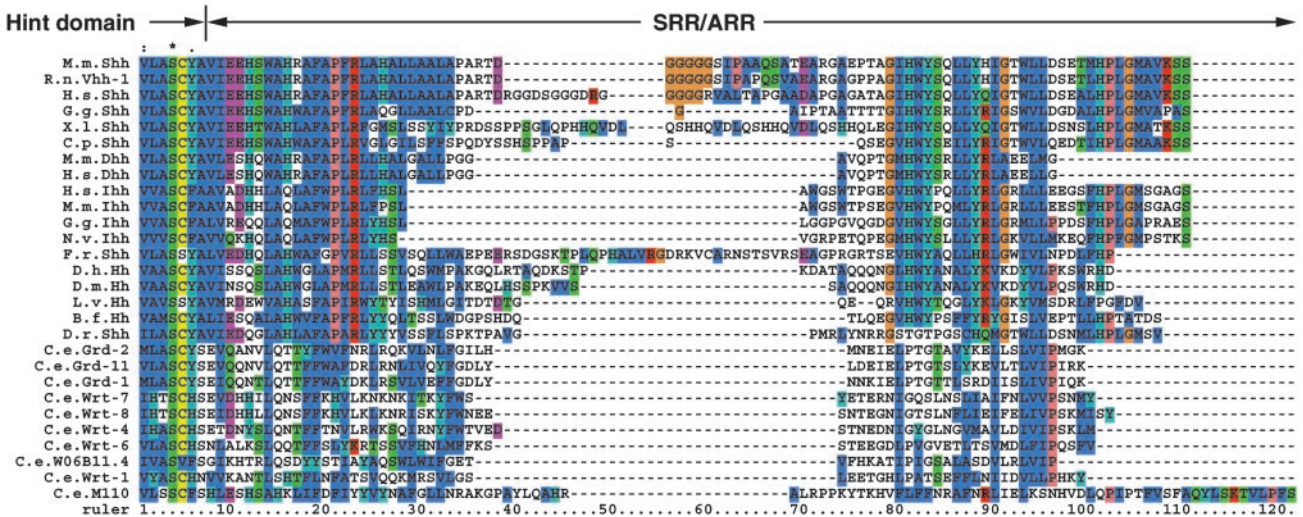


Figure 5 Sequence comparison of Hedgehog SRRs and the ARRs of nematodes. Using several residues of conserved sequence as an alignment anchor, the extreme C-terminal sequences of Hedgehog and nematode processing domains (i.e., the SRR and ARR domains, respectively) were aligned using the ClustalX algorithm (137). Species represented are, alphabetically, B.f., *Branchiostoma floridae* (Amphioxus); C.e., *Caenorhabditis elegans*; C.p., *Cynops pyrrhogaster*; D.h., *Drosophila hydei*; D.m., *Drosophila melanogaster*; D.r., *Danio rerio*; F.r., *Fugu rubripes*; G.g., *Gallus gallus*; H.s., *Homo sapiens*; L.v., *Lytechinus variegatus*; M.m., *Mus musculus*; N.v., *Notophthalmus viridescens*; R.n., *Rattus norvegicus*; and X.l., *Xenopus laevis.* Despite the lack of striking sequence conservation in corresponding segments from nematodes and the Hh proteins, some similarities, most notably the clustering and spacing of hydrophobic residues, are clear. This partial conservation, together with the ability of the *Drosophila* processing domain to utilize alternative steroidal compounds in the in vitro reaction, suggests that other sterols could be used in vivo to modify processed proteins. See Aspock et al. (52) for alignments of entire processing domains of Hedgehog and nematode proteins. Abbreviations are Shh, Sonic hedgehog; Ihh, Indian hedgehog; Dhh, Desert hedgehog; Grd, Ground; and Wrt, Wart.

with the amino-terminal domains of the Hh and nematode proteins. The sequence of events leading to formation of these proteins is not known. One possibility is that the Hint and SRR modules may have been assembled into a cholesterol transfer unit prior to association with the Hh signaling domain; alternatively, the Hint module might have been inserted within a preassembled protein comprising a signaling domain and the SRR precursor. In the second scenario, the SRR precursor in the preassembled protein might have served some function related to sterol recognition, such as membrane association. Similarly, several scenarios are possible in assembly of the nematode proteins. The possibility also exists that additional proteins will be found in which the Hint module initiates novel splicing or transfer reactions.

Amino-Terminal Acylation of Hedgehog Signaling Proteins

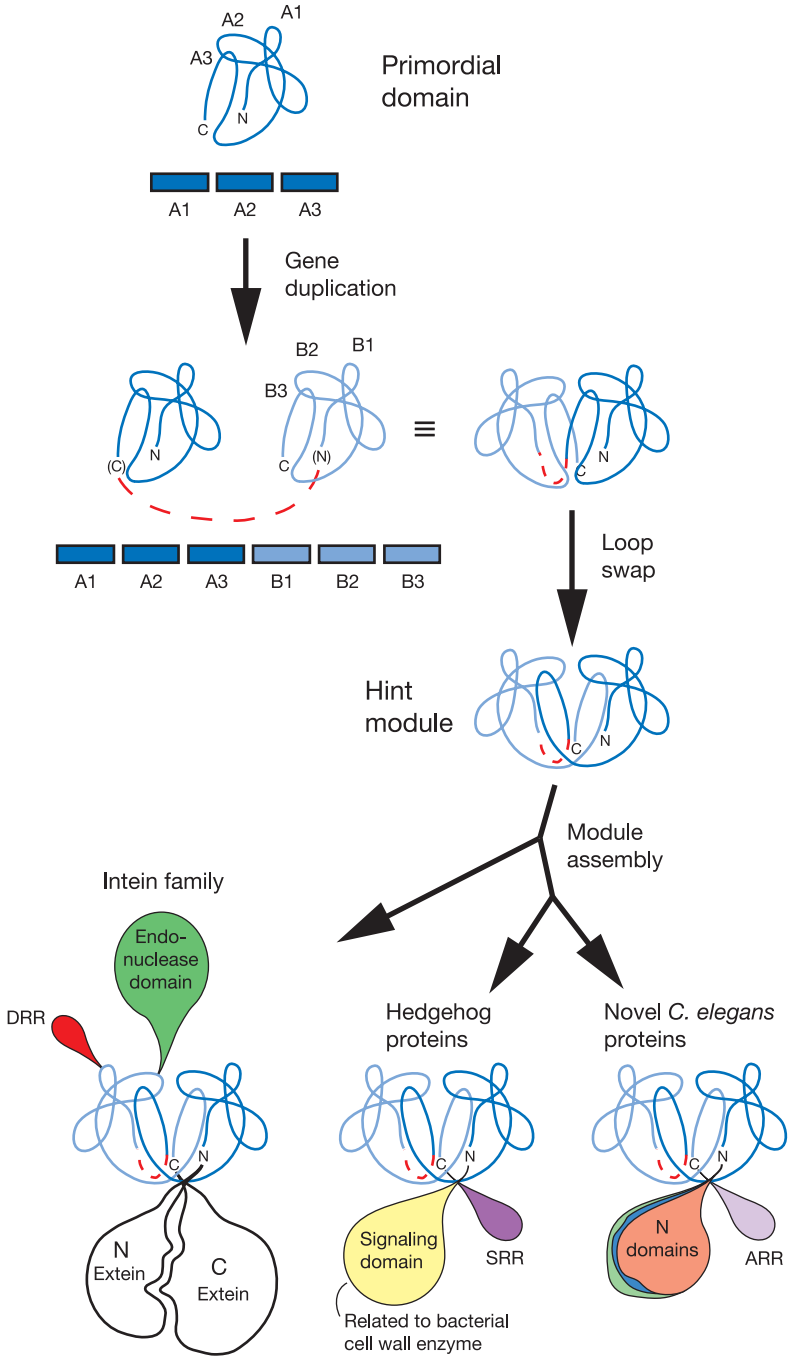
A second lipophilic modification of the Hedgehog signaling protein was more recently found to occur on a large proportion of the amino-terminal signaling domain of human Sonic Hedgehog upon expression in either insect or mammalian cell lines (57). This additional modifying adduct is a fatty acid, usually palmitate, and is found in an amide linkage with the amino-terminal cysteine that is exposed by signal sequence cleavage (see Figure 1*A*). Because this Cys residue is the first of a pentapeptide, CGPGR, that is widely conserved among species, there is a possibility that these residues and others nearby may constitute an important determinant for the palmitoylation reaction. The fatty acylation is proposed to occur via a thioester intermediate involving the side chain of the amino-terminal cysteine, followed by a spontaneous rearrangement to form the amide. The efficiency and specificity of this modification appears to depend in part upon prior cholesterol modification because the level of acylation is reduced and the types of modifying fatty acids are varied when the Shh protein is produced from a truncated construct lacking the processing sequences. When Shh is expressed in cultured mammalian cells using a moderately active (nonviral) promoter, we have observed that the majority of Shh-Np is found in a doubly lipidated form (58; R.K. Mann and P.A. Beachy, unpublished information), providing additional evidence that this is the predominant form of the signaling molecule in vivo.

Whereas N-terminal palmitate is dispensable in some assays of Hh signaling activity, it is now clear, from both animal models and cultured cell-based in vitro assays, that this modification critically contributes to full signal potency. In the responsive chondrogenic cell line C3H10T1/2, recombinant versions of human Shh-N featuring fatty acyl adducts of intermediate chain length were found to be 40- to 160-fold more potent than the corresponding cysteine-initiated protein lacking an N-terminal adduct (59). Replacing the initiating cysteine with the structural cognate serine produces an even weaker signal (10-fold lower than unmodified) and removal of 5–10 N-terminal residues severely impairs signaling potency ($>$500-fold lower) (60). These results suggest the possibility that some degree of acylation can occur after addition of protein to target cells if the

cysteine target for acylation remains in place. Notably, it was also observed that replacing cysteine with hydrophobic residues alone conferred enhanced potency (up to eightfold greater than unmodified) (59). This result, combined with others involving a variety of nonfatty acyl N-terminal adducts indicates that the enhancement of signaling activity can be attributed to a general hydrophobic effect rather than exclusive specificity for palmitate or even long-chain fatty acids. It is also interesting that, at least in this assay system, the fatty acylated versions of recombinant Shh lacking C-terminal cholesterol modification feature potencies comparable to that of the doubly-lipidated Shh-Np (produced from the full-length gene in metazoan cell lines).

The importance of Hedgehog palmitoylation has also been demonstrated in several animal models of development wherein modification site mutants were found to have little or no activity in vivo. Lee et al. (61) found that a transgene encoding a serine substitution at the palmitoylation site in *Drosophila* Hedgehog not only nullified its ability to complement loss-of-function alleles responsible for embryonic and larval defects but also caused a dominant negative effect with wild-type endogenous Hedgehog. A separate study (62) employing ectopic expression in mouse as well as tissue explant assays confirmed the necessity of N-terminal acylation and demonstrated that mutation of the acylation site caused a loss of Shh signaling activity, consistent with cultured cell-based in vitro observations. Furthermore, a *Shh* mutant allele encoding a truncated protein results in reduced signaling and defective limb patterning upon expression within the developing limb (63). The precisely truncated Shh-N protein produced by this allele is not autoprocessed, consequently lacks cholesterol, and has reduced

Figure 6 Possible evolutionary history of inteins, Hh proteins, and nematode proteins. Schematic drawings, based on the crystal structure of the Hedgehog C-terminal autoprocessing domain (51), illustrate an ancestral structure that gave rise to the Hint domain proteins through gene duplication, domain swapping, and insertion events, which may have occurred during evolution. The proteins formed by ligation of the N and C extein domains of intein family proteins are of many types with diverse biological activity. The Hh proteins feature a conserved N-terminal signaling domain that is related to a bacterial cell wall enzyme [see (138)], whereas the N-terminal domains of the nematode proteins are novel and sort into three groups (52). Module assembly refers to evolutionary pathways wherein Hint sequences, either alone or carrying an appended domain (e.g., an endonuclease), are inserted into an existing protein. The sequence of depicted events should be considered speculative. Possible scenarios for the assembly include prior association of the SRR with the Hint module to form an independent cholesterol transfer entity. Alternatively, the Hedgehog signaling domain may have evolved with the hydrophobic SRR domain, between which the Hint domain was later inserted. See text and (51) for discussion. An evolutionary dendrogram relating the various Hedgehogs and nematode proteins, which are more closely related, is presented in Aspock et al. (52). Abbreviations are SRR, sterol recognition region; Hint, Hedgehog intein; and DRR, DNA recognition region.



amino-terminal acylation (see below); a reduction in signaling and patterning activity of this mutant is consistent with a requirement for palmitoylation in producing a fully potent Shh signal.

Mechanism and Specificity of Hedgehog Palmitoylation

Most of what is known about the mechanism, specificity, and function of protein S-acylation derives from the numerous and well-documented examples of this dynamic cytoplasmic phenomenon that targets many key intracellular and transmembrane signaling proteins [see (64) and (65) for excellent recent reviews; also see Smotrys and Linder in this volume]. Palmitoylation of Hh proteins, however, is distinctive in that it takes place within the secretory pathway, presumably isolated from the machinery that generates and regulates other S-acylated proteins, and it resolves into a stable amide linkage (see below). Whereas Hedgehog palmitoylation is not the first example of protein S-acylation within the secretory pathway [apolipoprotein B palmitoylation has been documented and shown to be required for assembly of various serum lipoprotein particles; see (66)], it is especially intriguing given the complex biogenesis and signaling activities of Hh proteins.

Although nonenzymatic protein S-acylation has been observed in vitro and may have a specialized role within mitochondria (67), the likelihood of its significance as a general mechanism is minimal given the activity of acyl-CoA binding proteins that reduce the concentration of free acyl-CoA species to levels inadequate for such reactions [(68); also see (64)]. In addition, the recent purification and cloning of two protein acyltransferases (PATs) from *Saccharomyces cerevisiae* that act on cytoplasmic substrates have demonstrated that the acylated components of certain pathways (e.g., the Ras pathway in yeast) have dedicated enzyme-based machinery that is utilized in these reactions (69, 70). Whereas derivatization, including palmitoylation, of N-terminal cysteinyl residues of Hh proteins can also be achieved in vitro [see (59)], autoacylation of Hh in vivo is, likewise, unlikely to happen to any appreciable extent. Recent genetic screens for new patterning mutations in *Drosophila* have supported the idea of enzyme-catalyzed acylation by identifying a single, novel gene that is required for production of an active Hedgehog signal in vivo (71–74). Molecular characterization of this gene revealed that it encodes a protein belonging to the MBOAT family of enzymes (membrane-bound O-acyltransferases), some of which are known to catalyze esterification reactions involving, principally, lipids and other relatively small molecules (75). This insight lead to the obvious proposal that the gene, variously termed *sightless* (*sit*), *skinny hedgehog* (*ski*), *central missing* (*cmn*) and *rasp*, is responsible for the previously identified N-terminal palmitoylation. Genetic knockdown of the activity of *ski* (as we shall refer to this gene) by RNAi causes a reduction in overall Hedgehog protein hydrophobicity, as compared to the fully modified species, and to a level that matches that of the unacylated version (72). Although this reaction, thus far, has not been reconstituted in vitro, these results suggest that this putative acyltrans-

ferase is responsible for catalyzing the acylation of Hh proteins in vivo. Vertebrate forms of *ski* have been identified, and their activities, doubtless, will be examined in other models of development and evaluated as to whether they are required in different signaling pathways. In tests of acyltransferase activity with model, non-Hedgehog substrates, the Ski protein has, thus far, proven inactive (R.K. Mann and P.A. Beachy, unpublished information).

The precise mechanism whereby a putative O-acyltransferase accomplishes an amide-linked lipidation is still undefined, but a strict requirement for a cysteine at the site of modification is indicative of, in the early stages at least, a conventional protein S-acylation. In contrast to protein N-myristoylation, enzyme-mediated protein S-acylation, as understood for cytoplasmic proteins, takes place posttranslationally, requires at a minimum a free sulfhydryl acceptor moiety (i.e., an accessible cysteine), and, generally, a prior membrane-anchoring of the target protein through a region of the protein that is typically near the site of the modification. These membrane anchors can take many forms and include transmembrane polypeptide helices, N-terminal myristoyl moieties, C-terminal polyisoprenoid adducts, clusters of hydrophobic residues, as well as spans of basic residues (see above-mentioned reviews). Prior cysteinyl-isoprenylation at the C-terminal CaaX motif of Ras proteins, for example, is required prior to S-acylation at adjacent cysteines [(76), also see (77)]. These membrane anchors are thought to recruit the acylation targets to cellular membranes where most PATs have been shown to localize [see (65)]. Depending on the sequence of processing events, a number of motifs within Hh proteins might satisfy a membrane anchor requirement and include the cholesterol adduct itself (assuming prior internal processing), the polybasic cluster adjacent to the site of palmitoylation (see Figure 7), or the hydrophobic motif (see above) at the extreme C terminus of the Hedgehog processing domain (if N-terminal processing precedes internal). The hydrophobic signal sequence is unlikely to provide the anchoring activity because this is lost early due to cotranslational processing. As mentioned above, both the efficiency and the fatty acyl selectivity of Hedgehog S-acylation depends, to some degree, on the presence of the cholesteryl adduct because expression of Hedgehog signaling proteins lacking the cholesterol transferase domain causes a reduced level of palmitoylation as well as a greater variety of fatty acyl groups to be incorporated. This result suggests that the cholesteryl moiety facilitates membrane association and proximity to the Ski protein and that it does so in an organelle or compartment where palmitoyl-CoA is the predominant fatty acyl donor.

Regardless of the sequence, cysteine-initiated polypeptides, like Hh proteins after signal sequence cleavage, feature a free primary amine that is in an ideal position to attack the thioester and produce the more stable amide (see Figure 7). This S-to-N shift, which proceeds via a cyclic intermediate, has not only been observed in vitro but serves as the basis of expressed protein ligation [see above and (34)].

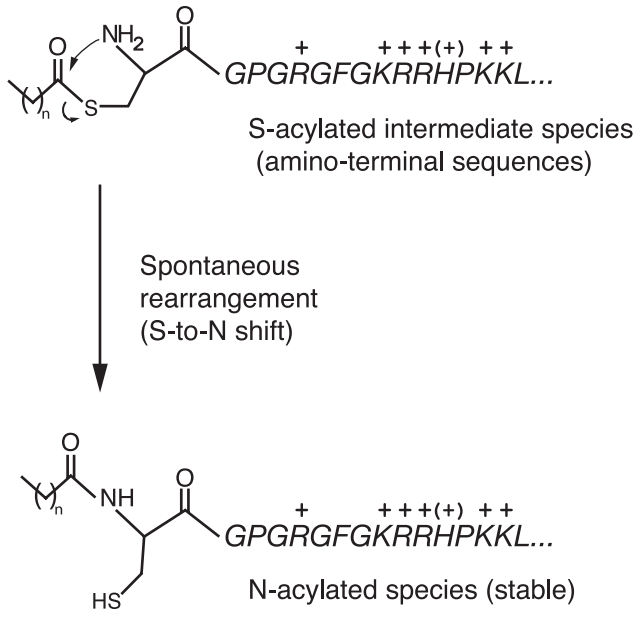


Figure 7 Model of the spontaneous acyl rearrangement at the amino terminus of Hh proteins. Palmitoylation of the amino-terminal cysteine residue likely occurs by conventional side chain S-acylation followed by a spontaneous S-to-N shift that results in the stable amide linkage. A polybasic cluster near the amino terminus of Hh proteins (the mouse Sonic Hedgehog sequence is shown) may serve to facilitate membrane association required during biogenesis and for distribution and activity of the signal. See text for details.

Functional Consequences of Palmitoylation

Although the determinants of protein S-acylation are still poorly defined, some of the consequences of these dynamic modifications are well documented. S-acylation, either on its own (at multiple sites) or in combination with other hydrophobic motifs (posttranslational modifications, hydrophobic amino acid residues, or hydrophobic associated proteins) serves a major role in targeting modified cytosolic proteins to specific membranes [see (64, 65, 77, 78)]. N-myristoylation of many Src family kinases alone, for example, provides only partial and transient association with cellular membranes. The addition of a palmitoyl moiety to the polypeptides (or, in the case of Src itself, the presence of a polybasic cluster), however, confers a strong membrane affinity in general and one that directs them specifically to the plasma membrane. Interestingly, this multiplicity of membrane targeting motifs appears to be the rule rather than the exception among anchored proteins. It is now generally accepted that any single acylation or prenylation is unable to confer stable membrane localization [(77–

79) and references therein], indicating that an additive or cooperative effect between intrinsic anchoring motifs drives the membrane localization. Even cholesterol-modified Hh proteins appear to be subject to the rule of cooperativity. Reducing N-terminal acylation of *Drosophila* Hedgehog in vivo (by RNAi-mediated knockdown of Ski activity; see above) causes a reduction in plasma membrane association as indicated by a significant increase in release of Hedgehog protein into the culture medium (72). This result has implications not only for Hedgehog protein generation and packaging but also in the distribution and reception of the Hedgehog signal (see below).

LIPID MODIFICATION IN THE WNT SIGNALING PATHWAY

The results of computational motif searching (75) used to implicate *ski* as a member of the MBOAT acyltransferase family also revealed the Wnt pathway gene *porcupine (porc)* as a member of this family. As shown for *ski*, *porc* is required in Wnt-producing cells for generating the fully functional protein signal (80, 81). Early reports suggested that Porc is required for Wnt protein secretion, and others have demonstrated that the activity influences the N-linked glycosylation status of the protein (82, 83). Willert et al. (84) recently reported the first purification of biologically active Wnt proteins and found, by metabolic labeling and with mass spectrometric-based mapping analyses, that both vertebrate and *Drosophila* Wnt proteins are modified by palmitate at a conserved cysteine residue. Mutation of this cysteine causes a loss of palmitate incorporation, and this mutation as well as enzymatic removal of the palmitate both reduce the biological activity of the protein. It remains to be demonstrated that Porcupine is directly responsible for Wnt protein palmitoylation, but considering available evidence, the connection appears likely.

It is possible that lipid modification will make another interesting entry into the Wnt pathway, this one at the level of extracellular signaling interactions. Among the many secreted antagonists of Wnt proteins [see (19)], the Dickkopf proteins have been found to possess a structure closely related to that of colipases (85). These proteins act as essential cofactors in the duodenal digestion of nutritional triglycerides by pancreatic lipase and bile salts. Colipases bind lipases, confer enhanced hydrophobicity, and are thought to recruit the enzyme complex to lipid-water interfaces [see (86)]. Although Dickkopf proteins have not been found in *Drosophila* and recruitment of a lipase to a Wnt-Dickkopf complex has yet to be demonstrated in vertebrate systems, hydrolysis of a lipid adduct from the Wnt protein could be the mechanism of Dickkopf inhibition of Wnt signaling.

HEDGEHOG TISSUE DISTRIBUTION

In addition to features intrinsic to the Hh proteins, a number of factors within both signal-generating and receiving tissues have been shown to influence the distribution and activity of the protein signal. Although dual lipidation of Hedgehog promotes membrane affinity, accessory proteins have evolved to deal with this fully processed form of the Hedgehog signal and are essential for its deployment in developing tissues.

The Role of Processing and Cholesterol Modification in Hedgehog Tissue Distribution

Despite its importance in embryonic pattern formation and in facilitating N-terminal acylation, the cholesterol adduct on Hh proteins is, paradoxically, not required for signal transduction through its receptors. Although aberrant in distribution, Hedgehog variants lacking the cholesterol moiety are able to signal to responsive tissues both close to and far from the source (32, 87). Similarly, as mentioned, some derivatives of recombinant Hedgehog that are only N-terminally modified are as active as dually lipidated forms (59). What is the mechanism whereby this adduct influences the proper deployment of Hedgehog signals? Given the overwhelming hydrocarbon content of cholesterol, coupled with the loss of its lone free polar moiety, one would expect that peptide cholesterylation would restrict the complex to the normal residence of a cholesterol molecule, i.e., the various membranes of the cell. This expectation has been borne out in several studies wherein processed, cholesterol-modified forms of Hedgehog have been found to be predominantly membrane-associated, and those lacking the adduct more freely dissociate from cells after secretion (22, 23, 57). The cholesteryl adduct thus functions as a lipid anchor that restricts the spatial mobility of this secreted signal. But processed Hedgehog protein travels beyond the cells in which it is produced and signals over many cell diameters [see (1, 88–91)]. This signaling is direct, rather than through an intermediary signal (92, 93), suggesting that cellular mechanisms may exist for the handling and delivery of cholesterol-modified proteins.

The Contrasting Roles of Dispatched and Patched in Tissue Distribution of the Hh Signal

Specific cellular activities dedicated to the handling of cholesterol-modified Hh proteins indeed have been found and feature distinct activities required in signal-generating and in signal-receiving cells. One of these activities was identified in a genetic screen for new mutations affecting Hedgehog signaling in *Drosophila* embryos and imaginal discs. The function of this gene is required exclusively in Hh-producing cells for release of a fully functional signal (87). In *dispatched* mutants Hh protein production and processing appear to be uncompromised, but the signal generated within mutant cells accumulates and does not

travel to distant targets. Interestingly, the gene is not required for release of a truncated form of Hh (i.e., one lacking the cholesterol adduct), suggesting that one of its normal functions is to make the cholesterol-modified version mobile. Dispatched was recently shown to be required in the *Drosophila* embryo for generating apically-localized, Hedgehog-containing punctate structures that are likely involved in many of the embryonic patterning activities of Hedgehog (94). Again, it is the cholesterol-modified forms of Hedgehog that are subject to the activity of Dispatched, confirming its role in mobilizing the anchored form of the signal. The Dispatched protein is predicted to contain 12 transmembrane segments and is related throughout the transmembrane region to a group of proteins that include bacterial transmembrane transporters and Patched, a receptor for the Hh protein (see below). A 5-transmembrane subset of this homology region is conserved in certain proteins that sense and regulate sterol homeostasis and is known as a sterol sensing domain (SSD) (see below).

The murine and human genomes contain two *Dispatched* homologs, and genetic studies in mice support an essential role for the function of one of these genes (*mDispA*) in Hh signaling (95). Homozygous loss-of-function *mDispA* mutations are embryonic lethal, and mutant embryos display an array of phenotypes consistent with a complete loss of Hedgehog pathway patterning activity (95–97). The severity of this phenotype suggests that Hh signaling function can be assigned predominantly to *mDispA*; this is consistent with the ability of *mDispA* but not *mDispB* to rescue *Drosophila disp* mutants.

Some insight into the biochemical function of Dispatched has been derived from cultured cell-based models of Hedgehog protein signal generation (95). Whereas expression of full-length Hh proteins normally results in a strong membrane association, coexpression with *Dispatched*, or *mDispA*, but not with *mDispB* results in a significant increase in Hh-Np and Shh-Np protein levels in the culture medium, indicative of an activity of Disp proteins in release of lipid-modified Hh proteins. In another study, a soluble form of modified Shh-Np was found to be released from mammalian cultured cells and suggested on the basis of gel filtration studies to exist in an aggregate of ~5–6 molecules (98), but the question of whether Dispatched activity was involved in producing this soluble aggregate was not addressed.

Dispatched as well as Patched proteins display topological and sequence similarity to the RND (for resistance, nodulation, division) family of bacterial transmembrane transporters. These permeases utilize a proton electrochemical gradient to function as antiporters in extruding from bacterial cells a variety of substrates that include heavy metals, hydrophobic drugs, and endogenous compounds [see (99)]. The structure of one member of this transporter family, AcrB, has been solved to atomic resolution (100), and the monomer comprises 12 transmembrane spans that appear to have arisen through a tandem duplication of a 6 transmembrane unit (Figure 8*A*). The similarity of Disp and Ptc to these transporters extends throughout the transmembrane region and is particularly striking in TM4, which contains a Gly-X-X-X-Asp motif (Figure 8*B*) that is

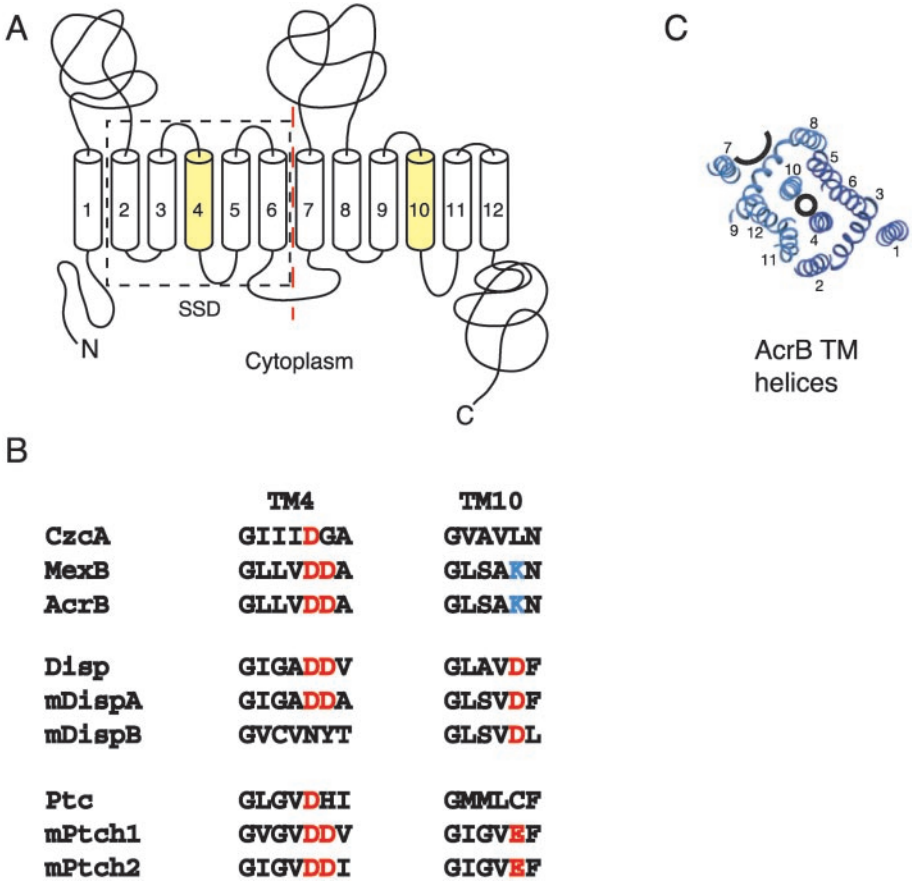


Figure 8 Structure and sequence conservation between bacterial RND transporters, Dispatched and Patched proteins. (*A*) Predicted topology of Dispatched proteins. This 12-span polytopic transmembrane model is also representative of other members of a class that includes Patched and the bacterial RND family of transport proteins. The five adjacent transmembrane segments that have been found to confer a sterol sensing activity (e.g., in HMG-CoA reductase and SCAP) are delimited by a dashed box. The depicted topology and domain structure is from sequence-based predictions reported by Burke et al. (87) and Ma et al. (95). Relative loop domain lengths are approximate; loop drawings are stylized and do not reflect known or predicted folds. A red dashed line divides the protein into two homologous spans that are likely the result of gene duplication. (*B*) Conserved sequences within TM4 and TM10 domains of Dispatched and Patched proteins as well as several bacterial transporters, which include AcrB. Note that TM4 of *mDispB*, which fails to rescue Drosophila *disp* mutations, does not contain the conserved Asp (D) residue present in other family members. (*C*) Cross section of transmembrane helices from the crystal structure of AcrB (100); only one subunit of the trimeric structure is shown. It has been proposed that the opening between the central TM4 and TM10 domains (note drawn *circle*) serves as the pore for proton translocation.

critical for function of the transporter in biochemical reconstitution experiments (101–103). TM4 and TM10 are positioned inside a transmembrane helix bundle (see Figure 8*C*) with the TM4 Asp residue forming a salt bridge with a Lys at the corresponding position in TM10, and these helices are proposed as candidates for the proton-translocating pathway.

Mutations of charged residues at these positions in TM4 and TM10 of Disp (95) and Ptc (see below) disrupt biological function, reinforcing the suggestion from sequence conservation that Disp and Ptc are functionally related to RND transporters and may act by similar mechanisms. Although the proposed export of a lipoprotein would represent a novel activity for an RND transporter family member, there is precedent for this function in members of the structurally distinct ATP binding cassette (ABC) family of exporters [see (104)]. It is also intriguing that AcrB appears to function as a trimer, raising the possibility that multimeric action of Disp protein may produce an aggregated, soluble form of the lipid-modified Hh proteins, perhaps a micelle-like structure with interactions between lipids forming a lipophilic interior. It remains to be seen whether the Dispatched-dependent export of Hh proteins is powered by a proton motive force, like the activities of other RND transporters, or by some other electrochemical gradient.

With possible relevance to Dispatched function, it is interesting to note that Hh-Np from *Drosophila* embryos partitions into detergent-insoluble glycolipid enriched complexes, as has been shown for other raft-associated proteins (105). In addition, murine Shh-Np also partitions with such complexes (58), suggesting that raft association is a general property of the processed Hh signal. It is not known whether raft association is conferred by cholesterylation or palmitoylation alone, or whether both modifications are required. Irrespective of how raft association is specified, it has potential consequences both for signal packaging from signal-producing cells and for signal response in target cells. It is possible that a raft-based process, conceivably involving Dispatched (see above), may operate in signal packaging and secretion of raft-targeted Hh proteins for long-range signaling. With regard to rafts in target cells, *dally-like* (*dlp*) encodes a GPI-linked protein required for response in target cells (106–108) and presumably would be found in rafts, as is the case for other GPI-linked proteins. Perturbations of cholesterol homeostasis, whether genetic or pharmacologic, can also disrupt Hh signaling through components that are unlikely to be involved with the production or distribution of the Hh signal (109). We have thus found that such perturbations disrupt signal response in receiving cells at the level of the seven transmembrane component Smoothened (110).

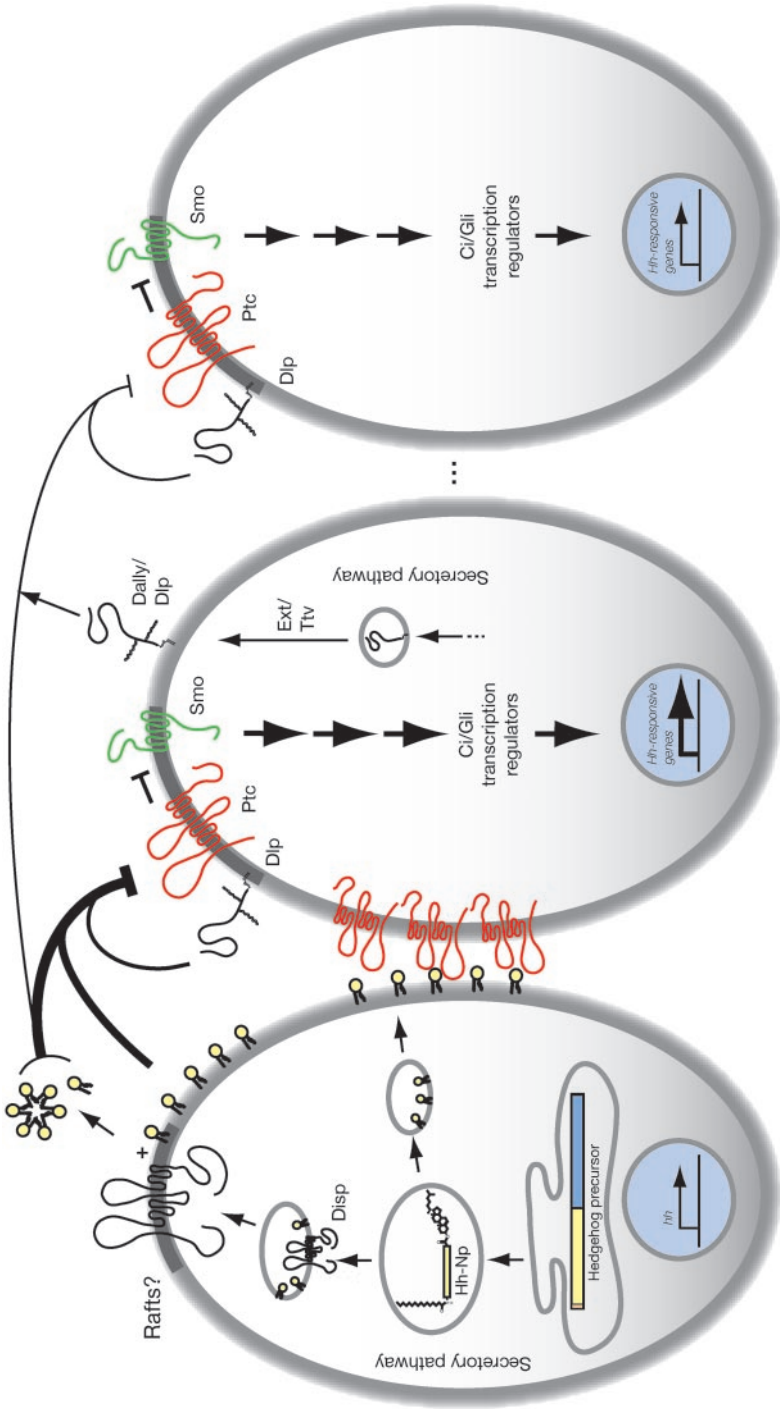
The Ptc protein itself plays a role in sequestration of the Hh signal within tissues, which is opposite to the role of Disp and is distinct from the better-known role of Ptc in regulating signal transduction as a component of the Hh receptor mechanism. The role of Ptc in Hh signal transduction is to functionally antagonize the activity of Smoothened, a multi-pass transmembrane protein, which in the absence of Ptc function constitutively activates the Hh pathway (111–114).

Ptc acts catalytically in its suppression of Smo activity (115), and disruption of Ptc function by mutation of the Gly-X-X-X-Asp motif in TM4 suggests that this catalysis may occur via a transporter-like activity. Ptc suppression of Smo activity is cell autonomous, and this suppression is alleviated by the presence of the Hh signal, which interacts directly with the Ptc protein (116–118).

A second activity of Ptc, which is more directly relevant to the current discussion, is its role in sequestration of the Hh signal within tissues, a cell non-autonomous activity that restricts the spatial extent of Hh signaling (112). The transduction and tissue distribution activities can be genetically uncoupled, as demonstrated by mutant proteins that retain either function in isolation. A mutant form of Ptc thus has been described that retains the sequestration function but is unable to suppress the Hh pathway (112, 119–121). Conversely, a mutant Ptc protein that fails to bind Hh but retains the ability to suppress Smo can be produced by deletion of a portion of the extracytoplasmic loop between TM7 and TM8 (93, 115). The sequestration and tissue restriction of a ligand by its receptor also has been noted for the Torso pathway in *Drosophila* (122). The sequestering action of Ptc appears to require a processed Hh protein as indicated by the observation that the spatial extent of signaling is greater when a truncated form of the Hh protein is produced at higher levels in its normal location (32). Ptc and Disp thus are proteins with homology throughout their predicted 12 transmembrane spans, and both proteins are required for appropriate tissue distribution of the processed, and therefore cholesterol-modified, form of the Hh protein. However, Disp mobilizes Hh-Np from signal-generating cells, whereas Ptc expression in adjacent tissues causes a striking limitation of that mobility (Figure 9).

The specific requirement of both Ptc and Disp for appropriate tissue distribution of the cholesterol-modified form of the Hh protein is an observation made more striking by the fact that both proteins contain a SSD (Figure 8*A*), which is a subset of the 12 transmembrane domains conserved between Ptc, Disp, and the RND transporters. The mechanism of sterol sensing remains unclear, but the

Figure 9 Short- and long-range Hedgehog signaling. Hedgehog-producing cells are known to signal to both adjacent as well as distant tissues. The described activities that impinge on short- and long-distance signaling, especially those pertaining to generation and distribution of cholesterol-modified Hh proteins, are depicted schematically. The figure integrates both genetic and biochemical relationships. The depicted multimeric form of secreted Hh protein has been proposed by Zeng et al. (98), but a requirement for Dispatched in its biogenesis has not been established. Darker portions of membrane illustrations suggest potential lipid raft/microdomain involvement. Hip1 (not shown) is a membrane-associated protein that is found only in vertebrates and, like Patched, is both induced by Hedgehog and known to restrict the movement of Hh protein by sequestration (139). Other abbreviations are Disp, Dispatched; Dlp, Dally-like protein; Ptc, Patched; Smo, Smoothened; Ttv, Tout velu; and EXT, Exostosin. See text for details.



SSDs of HMG-CoA reductase and SCAP allow these proteins to regulate their associated activities in response to cellular sterol levels (123–128). The obvious suggestion that SSDs may aid in binding of the cholesteryl adduct of Hh proteins is belied by the observation that the affinity of Shh-Np binding to Ptc is not significantly different from that of Shh-N, which lacks the cholesteryl adduct (57). Because Ptc and Disp appear to display a more extensive similarity to RND transporters throughout the transmembrane region, it appears likely that the SSD represents a functional subunit, perhaps a conformationally dynamic subdomain, of this larger region. In bacteria, which lack cholesterol, this subunit (the SSD) would have no function on its own, but in HMG-CoA reductase and SCAP, the SSD has perhaps been geared to respond to sterols.

The Role of Tout velu in Hedgehog Tissue Distribution

In contrast to the requirement for Dispatched in signal-generating tissues, a second gene, from yet another genetic screen in *Drosophila*, also causes a *hedgehog*-like phenotype but is required for signal transmission (94, 129, 130). This mutation, termed *tout velu* (or *ttv*, French for hairy), prevents normal propagation of the Hh signal in target tissue and allows signaling only within cells that directly abut the Hh-producing tissue. The normally processed form of Hh is restricted in the *ttv* mutants, whereas overexpression of a truncated, unmodified form of Hh is not, suggesting that the *ttv* gene product is required specifically to enable the transmission of the cholesterol-modified form of Hedgehog to distant targets.

Molecular cloning of *ttv* (130) revealed that it is a homolog of vertebrate *EXT-1*, a gene associated with hereditary multiple exostoses. EXT-1 and EXT-2 (another family member) are type II transmembrane proteins that form a Golgi-localized hetero-oligomeric complex, which has heparan sulfate copolymerase activity (131–133), and cells lacking EXT-1 function are defective in heparan sulfate proteoglycan biosynthesis. Confirming the structural similarity, it has also been shown that glycosaminoglycan, including heparan sulfate, biosynthesis is disrupted in *ttv* mutant animals (134–136). More recently, a requirement for Ttv activity in normal biosynthesis of the Dally and Dlp proteins has been demonstrated, and Dally and Dlp furthermore have been shown to function in mediating extracellular transmission of the Hh signal to distant targets (108). The role of Ttv in Hh signal distribution thus seems likely to be mediated through its action in biosynthesis of the Dally and Dlp proteins. Current information does not distinguish between direct function of the GAG chains elaborated by Ttv in Hh signal transmission or, instead, a requirement for GAG chain addition for proper surface presentation of the Dlp and Dally proteins. Interestingly, the Dlp protein appears to play roles both in autonomous cellular response to the Hh signal and in transmission of the Hh signal to distant sites (106–108).

PERSPECTIVE ON THE FUNCTION OF CHOLESTEROL MODIFICATION

Hh proteins are deployed in graded concentrations that are dependent not only on proximity to the source but also on influences from producing and receiving tissues. Although the precise mode of transmission through tissues is still unknown, it is likely that Hh proteins retain both of their relatively stable lipid adducts while en route to target tissues. Lipid modification would be expected to affect tissue distribution of Hh protein signals. Analysis of these effects with the use of truncated proteins, which lack cholesterol and therefore might be expected to travel more freely through tissues, is complicated by the accompanying reduction of amino-terminal acylation, which in turn reduces signaling potency (see above). However, when this reduction in signaling potency is compensated for by a higher-level expression of the truncated protein, it is evident that, in comparison to similarly expressed modified protein, Hedgehog protein unanchored by cholesterol is more mobile and acts over a greater range than the modified protein (32). Thus it seems clear that in addition to facilitating amino-terminal acylation and thus indirectly stimulating activity, the primary role of the cholesteryl adduct is to direct the mature signal to a set of cellular components that operate in concert to produce a precisely regulated distribution of Hh signals in responsive tissues. Paramount among a group of unresolved issues are the detailed roles of these lipid modifications in the intra- and extracellular packaging and handling of modified Hh protein, the mechanism by which these modifications modulate signal potency, and the role of such modifications in other extracellular signaling pathways, such as the Wnt pathway.

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