elements in the thorax. It may allow a systematic screen for identifying other such subdivisions that may underlie the development of the entire adult pattern.

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11. To obtain additional insertions, we used the same line carrying the *pGawB* transposon in the first chromosome described in (*8*). This insertion is homozygous lethal and is balanced with a *FM7* chromosome. *pGawB/FM7* females were crossed to y^-w^-; *DrP(Δ2-3)/TM6B* males carrying the source of transposase. The dysgenic F_1 females *pGawB/* y^-w^-; *P(Δ2-3)/+* were then crossed to y^-w^- males. The mobilization of the transposon resulted in y^-w^- (w^+) male progeny. Each of these F_2 males was individually crossed to y^-w^-; *UAS-y/TM3, UAS-y* females and the progeny were inspected under a dissecting microscope for y^+ patches.
12. X-Gal (5-bromo-4-chloro-3-indolyl β-D-galactopyranoside) staining in embryos was performed as described in (*8*), but sometimes we used antibody to β-galactosidase (anti–β-Gal) and standard methods. For better staining of adult cuticle with X-Gal, pharate adults were extracted from the puparium and treated as described in (*16*).
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Cholesterol Modification of Hedgehog Signaling Proteins in Animal Development

Jeffery A. Porter, Keith E. Young, Philip A. Beachy*

Hedgehog (Hh) proteins comprise a family of secreted signaling molecules essential for patterning a variety of structures in animal embryogenesis. During biosynthesis, Hh undergoes an autocleavage reaction, mediated by its carboxyl-terminal domain, that produces a lipid-modified amino-terminal fragment responsible for all known Hh signaling activity. Here it is reported that cholesterol is the lipophilic moiety covalently attached to the amino-terminal signaling domain during autoprocessing and that the carboxyl-terminal domain acts as an intramolecular cholesterol transferase. This use of cholesterol to modify embryonic signaling proteins may account for some of the effects of perturbed cholesterol biosynthesis on animal development.

The *hedgehog* (*hh*) family of secreted signaling proteins is required for developmental patterning of a wide variety of embryonic structures in insects, vertebrates, and other multicellular organisms (*1–5*). After signal sequence cleavage, the *hh* protein (Hh) precursor (~45 kD) undergoes an autocatalytic internal cleavage to yield an ~20-kD NH_2-terminal domain (Hh-N_p) and an ~25-kD COOH-terminal domain (Hh-C). Whereas Hh-N_p possesses all known signaling activity, Hh-C is responsible for precursor processing, acting by way of an intramolecular mechanism (*6, 7*). In addition to peptide bond cleavage, Hh autoprocessing causes the covalent attachment of a lipophilic adduct to the COOH-terminus of Hh-N_p (*8*). This modification is critical for the spatially restricted tissue localization of the Hh signal; in its absence, the signaling domain exerts an inappropriate influence beyond its site of expression (*8*). Physical and biochemical characterization of this lipophilic adduct indicates that it is not the glycosyl phosphatidylinositol (GPI) anchor (*8*), the only other known lipophilic modification associated with secreted cell surface proteins in eukaryotes (*9, 10*).

In vitro studies of Hh autoprocessing have used a bacterially expressed derivative of the *Drosophila* Hh protein, His_6Hh-C, in which most of the NH_2-terminal signaling domain and the signal sequence are replaced by a hexa-histidine tag. Cleavage of this protein occurs between residues corresponding to Gly^{257} and Cys^{258} (*7*) and likely proceeds through a labile thioester intermediate formed by the cysteine thiol and the glycine carbonyl carbon (*7, 8*). In the presence of high concentrations of thiols or other small molecules with strongly nucleophilic properties at neutral pH, cleavage of the peptide results from nucleophilic attack on the thioester carbonyl, causing displacement of the thiol group and formation of an adduct to Gly^{257} by the attacking nucleophile (*8*) (Fig. 1A). Thus, in reactions with 50 mM dithiothreitol (DTT), in vitro cleavage of His_6Hh-C proceeded to greater than 50% completion within 3 hours at 30°C (Fig 1B). At 1 mM DTT, however, the reaction yielded no visible cleavage product (Fig. 1B).

Howard Hughes Medical Institute, Department of Molecular Biology and Genetics, Johns Hopkins School of Medicine, Baltimore, MD 21205, USA.

*To whom correspondence should be addressed.

The in vivo reaction results in lipophilic modification of the NH_2-terminal signaling domain (*8*). The most direct mechanism by which this could occur, by analogy to the in vitro mechanism (Fig. 1A), would be for a lipid to function as the displacing nucleophile in attack of the thioester. To explore this possibility, we added bulk lipids extracted from *Drosophila* S2 cultured cells (*11, 12*) to the in vitro processing reaction in the presence of 1 mM DTT. Cleavage was observed, and the reaction proceeded to 20% completion in a 3-hour period (Fig. 1B). The reaction continues beyond this time and reaches ~50% completion by 18 hours (*13*). To identify the components active in the reaction, we separated the bulk S2 lipids into two classes, neutral and complex, by silicic acid column chromatography (*14*). The activity was found exclusively in the neutral class (*13*), so the lipids were subjected to preparative thin-layer chromatography (TLC) with a solvent system that resolves neutral lipids (*14*) (Fig. 2A). Lipid spots were visualized with iodine vapor or acid charring, and adsorbent at the corresponding positions of an identical uncharred plate was excised and extracted with chloroform-methanol-water. Only lipids extracted from spot B displayed stimulatory activity in the in vitro cleavage reaction (Fig. 2B).

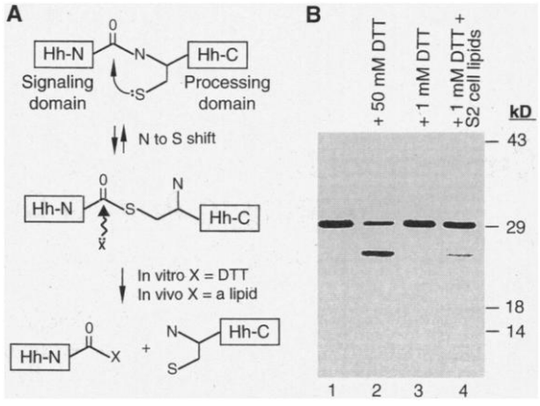

Fig. 1. Lipid stimulation of Hh autoprocessing in vitro. (**A**) Mechanism of Hh autoprocessing. The reaction is initiated by formation of a thioester between the thiol side chain of Cys^{258} and the carbonyl carbon of Gly^{257}, an N to S shift. This activated intermediate then undergoes a nucleophilic attack by DTT in vitro or by a lipophilic nucleophile in vivo, resulting in cleavage as well as formation of a covalent adduct at the COOH-terminus of the NH_2-terminal product. X denotes the attacking nucleophile. (**B**) Coomassie blue–stained SDS-polyacrylamide gel showing in vitro autocleavage reactions of the bacterially expressed His_6Hh-C protein (~29 kD) incubated for 3 hours at 30°C with no additions (lane 1), 50 mM DTT (lane 2), 1 mM DTT (lane 3), or 1 mM DTT plus bulk S2 cell lipids (lane 4). The Hh-C product of the autoprocessing reaction migrates as an ~25-kD species (lanes 2 and 4), and the ~5-kD NH_2-terminal product is not resolved in this gel.

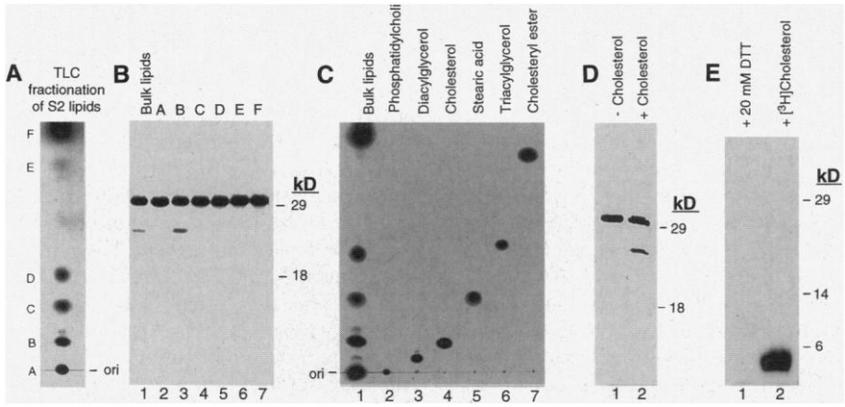

Fig. 2. Identification of cholesterol as the stimulatory lipid in the Hh autoprocessing reaction. (**A**) TLC plate coated with silica gel G (Merck) showing the fractionation of bulk S2 cell lipids with a heptane:ether:formic acid solvent (80:20:2). Six major spots are visualized by acid charring and are labeled A through F. (**B**) Coomassie blue–stained SDS-polyacrylamide gel showing in vitro autocleavage reactions of the bacterially expressed His_6Hh-C protein incubated with 1 mM DTT plus either unfractionated S2 cell lipids (lane 1) or lipids extracted from spots A through F (lanes 2 to 7, respectively). Addition of lipids from spot B, but not from any other spots, resulted in processing of His_6Hh-C protein. (**C**) TLC of S2 cell lipids (lane 1) along with selected lipid standards: phosphatidylcholine (lane 2), a diacylglycerol (lane 3), cholesterol (lane 4), stearic acid (lane 5), triacylglycerol (lane 6), and a cholesteryl ester (lane 7). Lipid spot B comigrates with cholesterol, as also demonstrated by mixing radiolabeled cholesterol with S2 lipids before TLC fractionation (*13*). (**D**) Coomassie blue–stained SDS-polyacrylamide gel showing that relative to 1 mM DTT alone (lane 1), cholesterol (0.35 mM) plus 1 mM DTT stimulates His_6Hh-C autocleavage in vitro (lane 2). (**E**) Autoradiogram of electrophoretically resolved products of His_6Hh-C autocleavage reactions driven by 20 mM DTT (lane 1) or 1 mM DTT plus 0.35 mM cholesterol (lane 2). Lane 1: [^{3}H]Cholesterol (3 μCi) was added at the end of the incubation period just before electrophoresis; lane 2: [^{3}H]cholesterol was present throughout the incubation period and is incorporated into the NH_2-terminal product of the reaction. To resolve the ~5-kD product of His_6Hh-C autocleavage, we separated reaction products in 17% SDS-polyacrylamide gels.

Using various lipid standards, we found that spot B comigrated with cholesterol (Fig. 2C). In addition, the active S2 cell–derived lipid displayed the same mobility as cholesterol in two other solvent systems and tested positive when sprayed with a specific reagent that reacts with sterols (*13–15*). Taken together, these results imply that the active lipid component is in the sterol fraction of the S2 lipids. Indeed, cholesterol, which is the principal sterol in eukaryotic cell membranes (*14*), displayed stimulatory activity similar to that observed with lipids extracted from spot B when added in pure form to the in vitro processing reaction (*16*) (Fig. 2D). To establish that the stimulatory activity of cholesterol is a result of its participation as a modifying group, we demonstrated that [^{3}H]cholesterol added to the 1 mM DTT reaction was incorporated into the NH_2-terminal product (Fig. 2E). No incorporation was seen, however, when [^{3}H]cholesterol was added just before electrophoresis to a reaction incubated for 3 hours with 20 mM DTT (Fig. 2E). Also consistent with covalent cholesterol addition, the NH_2-terminal fragment of His_6Hh-C generated by the cholesterol-driven reaction migrated just beneath the 6-kD marker, whereas the product of the reaction driven by 20 mM DTT migrated just above this marker (Fig. 3A). Such a shift in mobility, thought to result from an increased capacity for SDS binding to the covalently linked lipid (*17*), was also noted for Hh-N_p, as compared to the precisely truncated NH_2-terminal fragment (Hh-N, truncated after Gly^{257}) (*8*).

The part of the sterol most likely to act as attacking nucleophile is the 3β hydroxyl (see structure in Fig. 4B). Such an attack would leave cholesterol as a covalent adduct in ester linkage to the carboxylate of the terminal residue of the NH_2-terminal fragment (Gly^{257}). Ester bonds are subject to hydrolysis in alkaline conditions, and base treatment before electrophoresis indeed reduced the migration of the cholesterol-driven reaction product to a position coinciding with that of the DTT-driven reaction product (Fig. 3B). These results are consistent with stimulation of the in vitro processing reaction by direct nucleophilic attack of cholesterol on the thioester intermediate to form an ester-linked adduct. If

processing of Hh also results in formation of an ester-linked cholesterol adduct in vivo, then the protein-lipid linkage should be subject to base hydrolysis with a concomitant shift in electrophoretic mobility of the protein (normally 18.5 kD). The immunoblot in Fig. 3C shows the base-induced appearance of a species of reduced mobility (19.5 kD), which increased in abundance from ~one-third of the total after 5 min of treatment to most of the immunoreactive protein after 1 hour. This previously unidentified species comigrated with truncated, unprocessed Hh-N, which is not affected by base treatment. These data are consistent with an ester bond as the protein-lipid linkage in Hh-N_p.

To confirm the involvement of cholesterol in formation of the Hh-N_p adduct in vivo, we metabolically labeled S2 cells containing an inducible wild-type Hh construct with [^{3}H]cholesterol. After 48 hours of growth in the presence of [^{3}H]cholesterol, induced and uninduced cultured cells were detergent-extracted, and total cell proteins were subjected to SDS–polyacrylamide gel electrophoresis (PAGE) followed by fluorography (*18*). Whereas uninduced cells showed no incorporation of [^{3}H]cholesterol into cellular proteins, cells induced to express Hh showed a single strong band with a mobility corresponding to that of Hh-N_p (Fig. 4A). Given the hydrophobic character of Hh-N_p, these results suggest that either cholesterol itself or a sterol derivative constitutes the lipophilic adduct of Hh-N_p. To determine whether cholesterol is the final form of the adduct, we treated radiolabeled Hh-N_p protein excised from a gel with base to release the adduct, which was then isolated by ether extraction (*19*). Radiolabeled adduct was then subjected to analysis by high-performance liquid chromatography (HPLC) with a method designed to resolve various sterols (*20*) (Fig. 4B). The radioactive adduct released from Hh-N_p eluted at the same position as the cholesterol standard, and no radioactivity was detected in any other fraction (Fig. 4C).

The amount of radioactive cholesterol incorporated is consistent with that expected if all of the Hh-N_p synthesized upon induction receives a cholesterol adduct (*21*), suggesting that other cellular components do not compete effectively as nucleophilic adducts in the in vivo autoprocessing reaction. Also indicative of a homogeneous adduct, the mass of cholesterol is consistent with the mass previously measured by mass spectrometry of processed protein purified from cultured cells (*22*). These in vitro and in vivo results show that the Hh-C processing domain functions as a cholesterol transferase; as a result of this activity, a cholesterol adduct is attached via an ester linkage

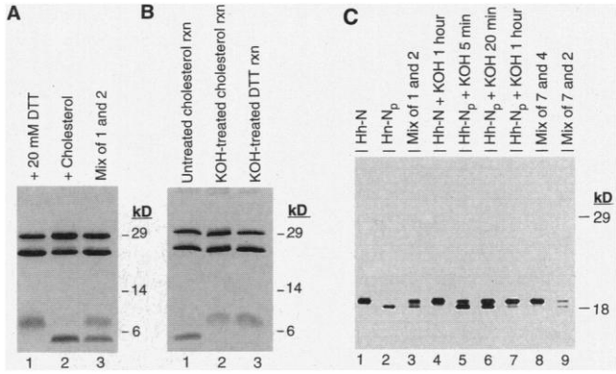

Fig. 3. Ester linkage of the adduct from Hh autoprocessing in vitro and in vivo. (**A**) Coomassie-stained gels of His_6Hh-C autocleavage reactions carried out in the presence of 20 mM DTT (lane 1) or 1 mM DTT plus 0.35 mM cholesterol (lane 2). Lane 3 contains a mixture of the samples loaded in lanes 1 and 2. The NH_2-terminal product of the cholesterol-driven reaction migrates ~2 kD faster than the DTT-driven reaction fragment. (**B**) Coomassie-stained gels showing protein products of His_6Hh-C autocleavage reactions carried out in the presence of 1 mM DTT plus 0.35 mM cholesterol (lanes 1 and 2) or with 20 mM DTT (lane 3). Before loading the gel, we incubated samples in lanes 2 and 3 for 60 min with 50 mM KOH in 90% methanol (*40*). Base treatment causes the cholesterol-driven NH_2-terminal reaction product to comigrate with the corresponding DTT-driven reaction product. (**C**) Autoradiogram of immunoblotted Hh NH_2-terminal domains purified from cultured S2 cells. NH_2-terminal domains were derived either from a construct truncated after Gly^{257} (Hh-N; lanes 1, 3, 8, and 9) or from a construct encoding wild-type Hh that produces the NH_2-terminal domain via the processing reaction (Hh-N_p, lanes 2 and 3 and lanes 5 to 9). Proteins were either directly loaded (lanes 1 and 2) or base-treated (*40*) for 5 min (lane 5), 20 min (lane 6), or 1 hour (lanes 7 and 4) before electrophoresis. Lane 3 contains a mixture of the samples loaded in lanes 1 and 2; lane 8 contains a mixture of the samples loaded in lanes 7 and 4; and lane 9 contains a mixture of the samples loaded in lanes 7 and 2. Upon base treatment, Hh-N_p undergoes a shift in mobility from 18.5 to 19.5 kD, the mobility of the unmodified Hh-N protein.

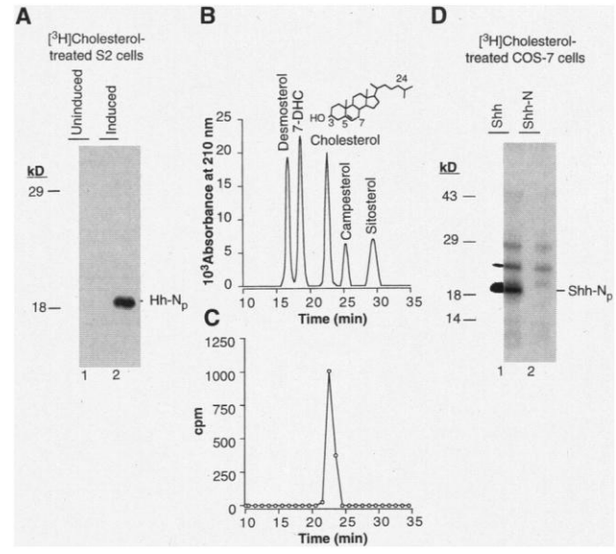

Fig. 4. Cholesterol as the in vivo adduct. (**A**) Autoradiogram of a gel loaded with total cell proteins from S2 cells containing a stably integrated Cu^{2+}-inducible *hedgehog* gene. Before harvesting, these cells were grown in media supplemented with [^{3}H]cholesterol in the absence (lane 1) or presence (lane 2) of 1 mM $CuSO_4$. [^{3}H]Cholesterol incorporation is dependent on Cu^{2+} induction (lane 2) and is restricted to a single protein species migrating at a position corresponding to that for Hh-N_p. (**B**) HPLC profile of sterols separated on a C18 column by isocratic elution with a solvent containing methanol:ethanol:water (86:10:4) (*20*). Approximately 5 μg of each sterol was mixed, loaded, and elution monitored by absorbance at 210 nm. The structure of cholesterol is shown above the cholesterol peak. Other sterols include desmosterol, which contains one additional double bond between carbons 24 and 25; 7-dehydrocholesterol (7-DHC), which contains one additional double bond between carbons 7 and 8; campesterol which contains an additional methyl group on carbon 24; and sitosterol, which contains an additional ethyl group on carbon 24. (**C**) HPLC analysis as in (B) of the adduct released by base treatment of Hh-N_p metabolically labeled with [^{3}H]cholesterol (A). The radioactive species recovered from the metabolically labeled protein coelutes with cholesterol. (**D**) Metabolic labeling of vertebrate *Sonic hedgehog* protein with [^{3}H]cholesterol. Autoradiogram of a gel loaded with total cell proteins from COS-7 cells transfected with a wild-type *Sonic hedgehog* expression construct (*Shh*, lane 1) or a construct that generates an unprocessed NH_2-terminal protein truncated after the conserved glycine at the site of autocleavage (*Shh-N*, lane 2). The COS-7 cells were incubated in culture medium supplemented with [^{3}H]cholesterol for 24 hours before and 36 hours after transfection (*23*). A strongly labeled species migrating at ~20 kD, corresponding to the mobility of Shh-N_p, is observed in cells transfected with the *Shh* but not the *Shh-N* construct. Less heavily labeled species are apparent in both lanes and may represent other cholesterol-modified proteins.

to the COOH-terminus of the NH_2-terminal signaling domain of the Hh protein.

To examine whether processing of vertebrate *hedgehog* proteins results in the incorporation of cholesterol as a covalent adduct to the signaling domain, we metabolically labeled cultured green monkey kidney cells (COS-7) with [^{3}H]cholesterol and transfected the cells with expression constructs containing (i) the full-length murine *Sonic hedgehog* (*Shh*) open reading frame, resulting in production of an autocatalytically processed signaling domain (Shh-N_p), or (ii) *Shh* coding sequences precisely truncated at the site of cleavage, thus producing an unprocessed NH_2-terminal signaling domain (Shh-N) (*23*). Cells expressing the full-length construct contained a prominent radiolabeled species migrating at ~19 kD, suggesting that cholesterol is covalently added to Shh-N_p (Fig. 4D). This band was not present in cultures expressing the truncated Shh-N protein (Fig. 4D), indicating that the incorporation of [^{3}H]cholesterol is dependent on the presence of the Shh processing domain. These data strongly suggest that the ability to attach cholesterol as a covalent adduct during autocatalytic processing and cleavage is a universal property of Hh proteins. Several other protein species in addition to the Shh NH_2-terminal domain also appeared to incorporate [^{3}H]cholesterol in cells transfected with either construct, suggesting that covalent modification by cholesterol extends to proteins beyond the Hh family. This possibility is consistent with the recently reported occurrence of several sequences homologous to the Hh processing domain in association with NH_2-terminal domain sequences distinct from hedgehog (*8*).

Cholesterol has been studied for over 200 years as a result of its unique biochemical properties, its role in vertebrates as a biosynthetic precursor of steroid hormones and bile components and, more recently, its role in human atherosclerotic disease (*24, 25*). Cholesterol has also been proposed as having a more general structural role in modulating the properties of biological membranes of animals (*24, 26*), but this is not a universal requirement because some animal groups such as insects and nematodes cannot synthesize cholesterol de novo and survive as adults when fed a sterol-free diet (*27–29*). Given that autoprocessing is required for Hh activity (*6, 7*), a more universal role for cholesterol in animals may be as a covalent adduct to the NH_2-terminal domain of Hh and perhaps other similarly processed proteins. Thus, in *Drosophila*, although cholesterol is not required for adult viability, it is required for fertility and for embryonic development, two requirements that may relate to the genetically established role of *hh* in oogenesis and embryogenesis (*1, 30*).

Perturbations of cholesterol biosynthesis are associated with profound developmental defects in vertebrate embryos. Treatment of rats in early pregnancy with Triparanol, AY 9944^R, or BM 15.766, three inhibitors of cholesterol biosynthesis, causes pronounced birth defects that include cyclopia, monorhinia, agenesis of the pituitary and other ventral neuronal cell types, and other variable manifestations of holoprosencephaly (*31–36*). These defects resemble the severe holoprosencephalic phenotype of embryos lacking a functional *Shh* gene (*3*). The ability of cholesterol synthesis inhibitors to phenocopy *Shh* mutant embryos suggests that the defects may result from inefficient *Shh* processing attributable either to a shortage of cholesterol or to interference by an accumulation of specific biosynthetic precursors. Alternatively, if processing proceeds, these precursors may alter the properties of the processed protein or of the lipid bilayers with which it associates. Consistent with these possibilities, the teratogenic effects of AY 9944^R are blocked by a hypercholesterolemic diet, which both increases the level of cholesterol and decreases the accumulation of these precursors (*34*).

Another link between cholesterol and vertebrate embryonic development is the cholesterol synthesis defect associated with the Smith-Lemli-Opitz syndrome (SLOS) (*37–39*). Estimates of the prevalence of this autosomal recessive disorder range as high as 1 in 9000 births (alive and stillborn), with a carrier frequency of 2%. Severe SLOS is characterized by some of the malformations associated with holoprosencephaly, including microcephaly, pituitary agenesis, and midline facial anomalies, as well as by defects of the heart, kidneys, pancreas, limbs, and genitals. The genetic defect in SLOS eliminates the activity of the last enzyme of the cholesterol biosynthesis pathway, 7-dehydrocholesterol-Δ^7-reductase. Cells from SLOS patients thus are unable to synthesize cholesterol efficiently and instead accumulate 7-dehydrocholesterol, the same biosynthetic precursor that accumulates in rats treated with AY 9944^R or BM 15.766 (*39*). The spectrum of malformations and the sterol profile of SLOS patients thus suggests a loss of *Shh* function. Not all of the defects in AY 9944^R–treated rats and SLOS patients are phenocopies of the *Shh* mouse mutant phenotype. These distinctive defects may result from abnormal steroid hormone biosynthesis or from a loss of function of other proteins (for example, Fig. 4D), including other *hedgehog* proteins (*4, 5*), which normally receive a cholesterol adduct.

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16. A cholesterol stock in H_2O (1 mg/ml) was prepared by sonication in a Branson bath sonicator before addition to the cleavage reaction [150 mM NaCl, 100 mM tris-HCl (pH 7.4), 1 mM Triton X-100, 1 mM DTT, and His_6Hh-C protein]. The in vitro processing reactions contain His_6Hh-C at ~3.5 μM. Cholesterol was added to the in vitro reaction at 350 μM, a concentration 100-fold times as great as that of His_6Hh-C, to drive the processing toward completion, thus allowing for detection of the products by Coomassie blue staining of gels. We find that this reaction proceeds equally well at 12 μM cholesterol, only a 3.4-fold excess of cholesterol compared with His_6Hh-C. Because cholesterol has a critical micelle concentration (CMC) of 25 to 40 nM [M. E. Haberland and J. A. Reynolds, *Proc. Natl. Acad. Sci. U.S.A.* **70**, 2313 (1973)], essentially all of the cholesterol added to the in vitro reaction would be expected to be in cholesterol–Triton X-100 micelles. Given that most proteins that catalyze reactions with lipids function at or near the lipid bilayer, the mole percent of cholesterol relative to other lipids or detergents in micellar form is perhaps a more relevant measurement of concentration in these assays [M. H. Gelb, M. K. Jain, A. M. Hanel, O. G. Berg, *Annu. Rev. Biochem.* **64**, 653 (1995)]. Because the CMC of Triton X-100 is 0.3 mM, 12 μM cholesterol coresponds to ~1.7 mol% in our assay. By increasing the Triton X-100 concentration to lower the cholesterol mol%, we find that the reaction proceeds at ~50% efficiency at a cholesterol concentration of 0.02 mol%. Substrate Michaelis constants (K_m's) expressed in mol% have been determined for enzymes that also utilize lipid alcohols as substrates. For example, diacylglycerol kinase has an apparent K_m for dioleoylglycerol of 0.92 mol% [J. P. Walsh and R. M. Bell, *Methods Enzymol.* **209**, 153 (1992)]. Although His_6Hh-C is not a true enzyme in the sense that it is consumed during

the reaction, its ability to operate at ~50% efficiency at 0.02 mol% of substrate and the fact that cholesterol has been measured at 6% by weight of total lipid in the endoplasmic reticulum of eukaryotic cells [B. Alberts, *et al.*, *Molecular Biology of the Cell* (Garland, New York, ed. 3, 1994)] suggests a physiologically relevant affinity of His_6Hh-C for cholesterol.

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18. Metabolic labeling of S2 cultured cells with [^{3}H]cholesterol was done essentially as described (*28*). Briefly, cells containing a stably integrated Cu^{2+}-inducible *hedgehog* gene were grown at 23°C for 2 weeks in Schneider cell media (Gibco) containing 5% fetal bovine serum depleted of lipoprotein (low-cholesterol media, ~20 μg of cholesterol per milliliter).These cells were then plated at 40% confluence onto two 35-mm tissue culture dishes (Nunc) in 1 ml of low-cholesterol media supplemented with 300 μCi of labeled cholesterol [1.2.6.7-^{3}H (N)] (65 Ci/mmol, NEN) giving a specific activity for cholesterol in this medium of ~5 Ci/mmol. After 24 hours (one doubling time), one plate of cells was induced to express Hh protein by the addition of $CuSO_4$ (final concentration, 1 mM). After an additional 24 hours, the cells from both dishes were harvested, then lysed in tris-buffered saline containing 1% Triton X-100, and total cell protein was precipitated with 5 volumes of cold acetone. The protein pellet was resuspended in 2% SDS in H_2O and reprecipitated with acetone several times to remove unincorporated radioactivity before loading onto SDS–polyacrylamide gels for analysis. Initial labeling experiments with 25 μCi of added cholesterol resulted in reduced incorporation by a factor of ~10.
19. HPLC analysis of the Hh-N_p adduct involved gel isolation of the radioactive band, KOH-methanol treatment of the band to break the ester linkage (*40*), followed by neutralization of the solution with acetic acid, drying in a Speedvac, resuspension in H_2O, and extraction of the hydrophobic radioactivity with ether. After evaporation of the ether, the sample was resuspended in isopropanol and applied to the C18 column for analysis (*20*).
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21. The specific activity of [^{3}H]cholesterol in the S2 cell labeling medium was ~5 Ci/mmol. Assuming after a 24-hour doubling time that this concentration approximately represents that within the S2 cell membrane, then any protein subsequently expressed and receiving cholesterol as an adduct would also be labeled at the same specific activity. As determined by standardized Coomassie blue staining, ~50 to 100 ng (2.5 to 5 picomol) of Hh-Np is produced by one 35-mm dish of S2 cells containing the Cu^{2+}-inducible Hh construct during 24 hours of induction with 1 mM $CuSO_4$ (*13*). This amount predicts that ~12.5 to 25 nCi (2.75×10^4 to 5.5×10^4 dpm) of radioactivity would be incorporated into Hh-N_p protein produced in our labeling experiment, assuming that the protein is cholesterol modified. Total incorporation of radioactivity into Hh-Np during the in vivo labeling experiment described above (*18*) was measured at ~5×10^4 dpm by excision and scintillation counting of an Hh-Np gel band.
22. A recent matrix-assisted laser desorption ionization (MALDI) mass spectral analysis (*8*) gave a mass of ~430 daltons for the Hh-Np adduct, ~9% larger than the mass of cholesterol (386.6 daltons). Detection of this modification required that Hh-N_p be treated with cyanogen bromide (CNBr) in 70% formic acid because full-length Hh-N_p could not be detected. The mass discrepancy noted above could be accounted for by the net addition of formic acid (45 daltons) during CNBr digestion. This reaction could involve the addition of H_2O across the 5,6 double bond of cholesterol, a common reaction of secondary alkenes in strong acids [R. T. Morrison and R. N. Boyd, *Organic Chemistry* (Allyn Bacon, Boston, ed. 3, 1973)], followed by esterification of formate via this newly formed alcohol [B. I. Cohen, G. S. Tint, T.Kuramoto, E. H. Mosbach, *Steroids* **25**, 365 (1975)]. To determine whether the sterol backbone could be modified by the CNBr treatment, we examined a positively charged cholesterol derivative (3β(*N*-(*N'*,*N'*-dimethylamino)ethanecarbamoyl)-cholesterol) (Sigma) detectable by MALDI. Incubation of this sterol derivative in 70% formic acid alone resulted in the addition of 45 mass units to the sterol (*13*), a mass consistent with the net addition of a formic acid molecule.
23. COS-7 cells grown at 37°C in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum were plated at ~35% confluence onto two 35-mm dishes in 1 ml of Optimem media (Gibco) containing 1.5% fetal bovine sera and 250 μCi of [^{3}H]cholesterol, giving a final concentration of cholesterol of ~40 μg/ml with a specific activity of 2 Ci/mmol (labeling medium). After 24 hours the labeling medium was removed and the cells were transfected for 6 hours with *Shh* or *Shh-N* expression constructs with lipofectamine (Gibco) and serum-free DMEM. After transfection, 1 ml of fresh labeling medium was added to each dish, and the cells were incubated for 36 hours at 37°C. The cells were then harvested without washing and lysed on the plate with tris-buffered saline plus 1% Triton X-100, and the total cell proteins were precipitated with acetone, washed, and analyzed as described above for the S2 cell proteins.
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Gastric Mucosa Abnormalities and Tumorigenesis in Mice Lacking the pS2 Trefoil Protein

Olivier Lefebvre, Marie-Pierre Chenard, Régis Masson, José Linares, Andrée Dierich, Marianne LeMeur, Corinne Wendling, Catherine Tomasetto, Pierre Chambon, Marie-Christine Rio*

To determine the function of the pS2 trefoil protein, which is normally expressed in the gastric mucosa, the mouse *pS2* (*mpS2*) gene was inactivated. The antral and pyloric gastric mucosa of *mpS2*-null mice was dysfunctional and exhibited severe hyperplasia and dysplasia. All homozygous mutant mice developed antropyloric adenoma, and 30 percent developed multifocal intraepithelial or intramucosal carcinomas. The small intestine was characterized by enlarged villi and an abnormal infiltrate of lymphoid cells. These results indicate that *mpS2* is essential for normal differentiation of the antral and pyloric gastric mucosa and may function as a gastric-specific tumor suppressor gene.

The human (hpS2) (*1*) and mouse (mpS2) (*2*) pS2 proteins belong to the family of trefoil peptides, which are characterized by the presence of one to six cysteine-rich P domains (*3*, *4*). Although hpS2 and mpS2 are normally expressed in the gastric mucosa (*2*, *5*), hpS2 is also abnormally expressed in ulcerative gastrointestinal diseases (*6*) and in various cancers (*7*–*9*). In all cases, hpS2 and mpS2 are found in the cytoplasm of epithelial cells (*2*, *9*). It has been proposed that pS2 functions as a growth factor, a protease inhibitor, or a mucin stabilizer to modulate cell growth and protect the integrity of the gastric mucosa (*9*, *10*). To elucidate the function of pS2, we disrupted the mouse *pS2* gene by homologous recombination.

We cloned and sequenced the *mpS2* gene (*11*). It encompasses 4.1 kb and contains three exons (Fig. 1A). Exon 1 [96 base pairs (bp)] encodes the NH_2-terminal signal peptide, exon 2 (153 bp) encodes the P domain,

O. Lefebvre, R. Masson, J. Linares, A. Dierich, M. LeMeur, C. Wendling, C. Tomasetto, P. Chambon, M.-C. Rio, Institut de Génétique et de Biologie Moléculaire et Cellulaire, CNRS/INSERM/Université Louis Pasteur/Collège de France, Boite Postale 163, 67404 Illkirch Cedex, Communauté Urbaine de Strasbourg, France.
M.-P. Chenard, Service d'Anatomie Pathologique Générale, Centre Hospitalier Universitaire de Hautepierre, 67098 Strasbourg Cedex, France.

*To whom correspondence should be addressed.