Identification of a Palmitic Acid-modified Form of Human Sonic hedgehog*

(Received for publication, December 22, 1997, and in revised form, March 24, 1998)

R. Blake Pepinsky‡‡, Chenhui Zeng‡, Dingyi Wen‡, Paul Rayhorns‡, Darren P. Baker‡‡, Kevin P. Williams‡, Sarah A. Bixler‡, Christine M. Ambrose‡, Ellen A. Garber‡, Konrad Miatkowski‡, Frederick R. Taylor‡‡, Elizabeth A. Wang¶, and Alphonse Galdes‡

From ‡Biogen, Inc., Cambridge, Massachusetts 02142 and ¶Ontogeny Inc., Cambridge, Massachusetts 02139

During hedgehog biosynthesis, autocatalytic processing produces a lipid-modified amino-terminal fragment (residues 24–197 in the human Sonic hedgehog sequence) that is responsible for all known hedgehog signaling activity and that is highly conserved evolutionarily. Published in vitro biochemical studies using Drosophila hedgehog identified the membrane anchor as a cholesterol, and localized the site of attachment to the COOH terminus of the fragment. We have expressed full-length human Sonic hedgehog in insect and in mammalian cells and determined by mass spectrometry that, in addition to cholesterol, the human hedgehog protein is palmitoylated. Peptide mapping and sequencing data indicate that the palmitoyl group is attached to the NH2 terminus of the protein on the α-amino group of Cys-24. Cell-free palmitoylation studies demonstrate that radioactive palmitic acid is readily incorporated into wild type Sonic hedgehog, but not into variant forms lacking the Cys-24 attachment site. The lipid-tethered forms of hedgehog showed about a 30-fold increase in potency over unmodified soluble hedgehog in a cell-based (C3H10T1/2 alkaline phosphatase induction) assay, suggesting that the lipid tether plays an important role in hedgehog function. The observation that an extracellular protein such as Shh is palmitoylated is highly unusual and further adds to the complex nature of this protein.

The hedgehog proteins are a family of extracellular signaling proteins that regulate various aspects of embryonic development both in vertebrates and in invertebrates (for reviews, see Refs. 1 and 2). The most extensively characterized mammalian homolog is Sonic hedgehog (Shh),1 which is involved in diverse embryonic induction events, including the induction of floor plate and establishment of ventral polarity within the central nervous system as well as proper anterior-posterior patterning of the developing limb (3–6). In mediating these effects, Shh is believed to act both as a short range, contact-dependent inducer and as a long range, diffusible morphogen. Shh is expressed in the embryonic notochord, and induces floor plate formation at the ventral midline of the neural tube in a contact-dependent manner (3, 5, 6). Data suggest Shh can also act as a long range, diffusible morphogen, to promote subsequent differentiation of ventral neurons in a region-specific manner; e.g., dopaminergic neurons in the midbrain (7) and motor neurons in the spinal cord (6). It is presently unclear whether the same molecular species of Shh mediates both of these effects. While the mechanism of action of hedgehog proteins is not fully understood, biochemical and genetic data suggest that the hedgehog receptor is the product of the tumor suppressor gene patched (8–10), and that other proteins, including smoothened (9, 11), Cubitus interruptus or its mammalian counterpart gli (12, 13), and fused (14), are involved in the hedgehog signaling pathway.

Shh is synthesized as a 45-kDa precursor protein that is cleaved autocatalytically to yield a 20-kDa NH2-terminal fragment that is responsible for all known hedgehog biological activity (residues 24–197 in the human gene sequence) and a 25-kDa COOH-terminal fragment that contains the autoprocessing machinery (15–17). The NH2-terminal fragment remains membrane-associated through the addition of a lipid tether at its COOH terminus (18, 19). This tether is critical for restricting the tissue localization of the hedgehog signal. Recent biochemical data have identified the lipid tether as a cholesterol (18), the addition of which is catalyzed by the COOH-terminal domain during the autoprocessing step. Here we expressed full-length human Shh in a variety of systems and in characterizing the proposed NH2-terminal fragment, determined that cholesterol alone could not account for the increased mass of the membrane-tethered form of the protein. We identified a second lipid modification, a palmitoyl group, attached at its NH2 terminus. Along with the cholesterol, the palmitic acid modification is likely to have evolved as part of the mechanism for regulating short range-long range signaling by hedgehog.

EXPERIMENTAL PROCEDURES

Expression of Human Sonic hedgehog—The cDNA for full-length human Shh was provided as a 1.6-kilobase EcoRI fragment subcloned into pBluescript SK+ (20) (a gift of David Bumcrot from Ontogeny). 5′ and 3′ NolI sites immediately flanking the Shh open reading frame were added by unique site elimination mutagenesis using a Pharmacia kit following the manufacturer’s recommended protocol. The 1.4-kilobase NolI fragment carrying the full-length Shh cDNA was then subcloned into the insect expression vector, pFastBac (Life Technologies, Inc.). Recombinant baculovirus was generated by using the procedures supplied by Life Technologies, Inc. The resulting virus was used to create a high-titer virus stock. Methods used for production and purification of Shh are described below. The presence of membrane-associated Shh was examined by FACS and Western blot analysis. Peak expression occurred 48 h post-infection. For Western blot analysis, supernatants and cell lysates from Shh-infected or uninfected cells were subjected to SDS-PAGE on a 10–20% gradient gel under reducing conditions, transferred electrophoretically to Immobilon-P (Millipore),
and the Shh detected with a rabbit polyclonal antiserum raised against an NH2-terminal Shh 15-mer peptide-keyhole limpet hemocyanin conjugate. The cell lysates were made by incubating the cells for 5 min at 25 °C in 20 mM NaH2PO4, pH 6.5, 1% Nonidet P-40, 150 mM NaCl or in 20 mM Tris-HCl, pH 8.0, 50 mM NaCl, 0.5% Nonidet P-40, 0.5% sodium deoxycholate. The cell lysates were centrifuged at 10,000 g for 10 min at 4 °C in an Eppendorf centrifuge.

For expression of full-length Shh in mammalian cells, the 1.4-kilo- base NotI fragment containing full-length Shh was cloned into a derivative of the pCEP4 (Invitrogen) vector, CH269 (21). The construct was transfected into EBNA-293 cells using LipofectAMINE (Life Technologies, Inc.) and the cells were harvested 48 h post-transfection. The expression of surface Shh was verified by FACS and Western blot analysis. A chimeric gene product in which the NH2-terminal fragment (residues 24–197) of human Shh had been fused to the hinge and CH2, CH3 regions of human IgG, was expressed in Chinese hamster ovary cells.

Other constructs encoding soluble human Shh (residues 24–197 without the lipid modification), a mutant version of soluble human Shh containing a Cys-24 to Ser point mutation (C24S), and soluble human Indian hedgehog (Ihh, residues 28–202 in the human gene sequence) were expressed in Escherichia coli as His-tagged fusion proteins with an enterokinase cleavage site immediately adjacent to the start of the mature sequence. All of the constructs were confirmed by DNA sequencing. The fidelity of the final products after removal of the His tag with enterokinase (Life Technologies, Inc.) dissolved by NH2-terminal Sepharose 4B, was confirmed by electrospray ionization-mass spectrometry (ESI-MS). The cDNA encoding the NH2-terminal fragment of rat Sonic hedgehog (residues 25–198 in the rat gene sequence) was cloned into the baculovirus expression vector pBluebac III (Invitrogen) and expressed in insect cells essentially as described for the human full-length construct. The NH2-terminal fragment of rat Sonic hedgehog starts at Cys-25 and not at Cys-24 as in the human protein, since the signal sequence of the rat protein contains an additional amino acid residue. The rat sequence (residues 25–198) differs from the human sequence (residues 24–197) by only two residues; Ser-67 and Gly-196 in the human protein are replaced in the rat gene sequence (21).

Purification of Membrane-tethered Human Sonic hedgehog—The membrane-tethered form of Shh was produced in High Five™ insect cells (Invitrogen) using the recombinant baculovirus encoding full-length Shh discussed above. High Five cells were grown at 28 °C in SF-900 II serum-free medium (Life Technologies, Inc.) in a 10-liter bioreactor controlled for oxygen. The cells were infected in late log phase with about 2 × 107 cells/ml with virus at a multiplicity of infection of 3 or 5 (depending on the viability of the cell line at harvest was >50%). The cells were centrifuged and washed in 10 mM NaH2PO4, pH 6.5, 150 mM NaCl, 0.5 mM phenylmethylsulfonyl fluoride, the resulting cell pellet (150 g wet weight) was suspended in 1.2 liters of 10 mM NaH2PO4, pH 6.5, 150 mM NaCl, 0.5 mM phenylmethylsulfonyl fluoride, 5 μM pepstatin A, 10 μg/ml leupeptin, 2 μg/ml E64, and 120 μl of a 10% solution of Triton X-100. The cell lysate was subjected to a 30 min incubation at 70 °C. The particulates were removed by centrifugation (1500 × g, 10 min). All subsequent steps were performed at 4–6 °C. The pH of the supernatant was adjusted to 5.0 with a stock solution of 0.5 mM MES, pH 5.0 (50 mM final), and loaded onto a 150-μl SP-Sepharose FF column (Pharmacia). The column was washed with 300 ml of 5 mM NaH2PO4, pH 5.5, 150 mM NaCl, 0.5 mM phenylmethylsulfonyl fluoride, 0.1% Nonidet P-40, then with 200 ml of 5 mM NaH2PO4, pH 5.5, 300 mM NaCl, 0.1% Nonidet P-40, and bound hedgehog eluted with 5 mM NaH2PO4, pH 5.5, 800 mM NaCl, 0.1% Nonidet P-40. The Shh was next subjected to immunoaffinity chromatography on a mAb 5E1-Sepharose resin that was prepared by conjugating 4 μg of the anti-hedgehog antibody (27) per ml of CNBr-activated Sepharose 4B resin. The SP-Sepharose elution pool was diluted with 2 volumes of 50 mM HEPS pH 7.5, and batch loaded onto the 5E1 resin (1 h). The resin was collected in a column, washed with 10 column volumes of phosphate-buffered saline, 0.1% hydrogenated Triton X-100 (Calbiochem), and eluted with 25 mM NaH2PO4, pH 3.0, 200 mM NaCl, 0.1% hydrogenated Triton X-100. The elution fractions were neutralized and analyzed as described above, pooled, filtered through a 0.2-micron filter, aliquoted, and stored at −70 °C.

For metabolic labeling studies, High Five cells were seeded into T-75 flasks and infected with recombinant baculovirus encoding full-length Shh or with a control virus. 28 h post-infection the culture medium was changed and the cells were treated for 1 at 4 °C with 25 μCi/ml of 5E1-Sepharose and the other half with a control mAb-Sepharose of the same isotype. The resin were collected, washed three times with lysis buffer containing 0.2% Triton X-100, and treated with electrophoresis sample buffer. The samples were subjected to SDS-PAGE on a 10–20% gradient gel, and visualized by fluorography (3-day exposure).

Analysis of Tethered Human Sonic Hedgehog by Mass Spectrometry—Aliquots of Shh were subjected to reverse phase HPLC on a C4 column (Vydac, catalog number 214TP104, column dimensions 0.46 cm inner diameter by 25 cm) at ambient temperature. Bound components were eluted with a 30-min 0–70% gradient of acetonitrile in 0.1% trifluoroacetic acid at a flow rate of 1.4 ml/min. The column effluent was monitored at 280 nm and 0.5-min fractions were collected. 25–μl aliquots of fractions containing protein were dried in a Speed Vac concentrator. The dried samples were redissolved in 35 μl of either water or 1% trifluoroacetic acid and analyzed by ESI-MS. Fractions containing hedgehog were pooled, concentrated 4-fold in a Speed Vac concentrator, and the protein content calculated from the absorbance at 280 nm using a molar extinction coefficient of 26,030 liter mol⁻¹ cm⁻¹ that was calculated from the tyrosine and tryptophan content of Shh. Samples were subjected to ESI-MS on a Micromass Quattro II triple quadrupole mass spectrometer, equipped with an electrospray ion source. A volume of 6 μl (1 pmol/μl) of HPLC-purified hedgehog was infused directly into the ion source at a rate of 10 μl/min using 50% water, 50% acetonitrile, 0.1% formic acid as the solvent in the syringe pump. Scans were acquired throughout the sample infusion. All electrospray mass spectral data were acquired and stored in profile mode and were processed using the Micromass MassLynx data system.

Peptides from an endoproteinase Lys-C digest of pyridylethylated Shh were analyzed by reverse phase HPLC on-line with a Micromass Quattro II triple quadrupole mass spectrometer. The digest was separated on a Reliasil C18 column using a Michrom Ultrfast Microprotein Analyzer system at a flow rate of 50 μl/min with a 25-min 5–85% acetonitrile gradient in 0.05% trifluoroacetic acid. Scans were acquired from m/z 400 to 2000 throughout the run and processed as described above.

Sequencing of the tethered Shh was performed by post-source decay (PSD) measurement (22, 23) on a Voyager-DE™ STR (PerSeptive Bio-systems, Framingham, MA) matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometer using α-cyano-4-hydroxy-cinnamic acid as the matrix. 25 μl (1 pmol) of HPLC-purified endoproteinase Lys-C peptide was mixed with 0.5 μl of matrix (10 mg/ml in 50% acetonitrile) on the target plate. To cover the entire spectrum of fragment ions, the mirror voltages were decreased from 20 to 1.2 kv in 11 steps.

Cell-free Labeling with [3H]Palmitic Acid—Soluble human Shh was labeled with [3H]palmitic acid in a cell-free system by using a modified version of a published procedure (25). A crude microsomal fraction from rat liver was prepared by subjecting a liver homogenate to sequential centrifugation at 3,000 × g for 10 min, 9,000 × g for 20 min, and 100,000 × g for 30 min. The 100,000 × g pellet was suspended in 10 mM HEPS pH 7.4, 10% sucrose and again pelleted at 100,000 × g for 20 min. The final pellet (derived from 1 g of liver) was suspended in 3 ml of 20 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1 mM EDTA, 10 μg/ml leupeptin, and 0.5 μg of [9,10-3H]palmitic acid (50 Ci/mmol; NEN Life Science Products) were performed at room temperature for 30 min. Reactions were stopped with reducing electrophoresis sample buffer. The samples were subjected to SDS-PAGE on a 10–20% gradient gel, and visualized by fluorography.

Alkaline Phosphatase Induction in C3H10T1/2 Cells—Shh was tested for function in a cell-based assay measuring alkaline phosphatase induction in C3H10T1/2 cells (26) with a 5-day readout. The assay was performed in a 96-well format. Samples were run in duplicate. For tethered Shh (100 μg/ml), the samples were first diluted 200-fold with...
was stained with Coomassie Blue (from High Five insect cells and analyzed by SDS-PAGE. The protein Shh, prestained high molecular weight markers; lane b, soluble Shh (0.6 μg); lane c, tethered Shh (0.6 μg); lane d, mixture of soluble plus tethered Shh (0.6 μg each)). The ability of Shh to be modified with palmitic acid was assayed using a cell-free system described under “Experimental Procedures.” Soluble forms of hedgehog protein (3 μg/sample) were incubated for 1 h with rat liver microsomes, ATP, coenzyme A, and [3H]palmitic acid and then analyzed for palmitoylation by SDS-PAGE. The samples shown in lanes e-i were visualized by fluorography (lane e, Shh; lane f, des-1–10 Shh; lane g, C24S Shh; lane h, Ihh; lane i, His-tagged Shh) and in lanes j and k by Coomassie staining (lane j, Shh; lane k, des-1–10 Shh).

normal growth medium then subjected to serial 2-fold dilutions down the plates. Wells were normalized for potential effects of the added octylglucoside by including 0.005% octylglucoside in the culture medium. Blocking studies using the neutralizing mouse monoclonal antibody 5E1 (27) were performed by mixing Shh with serial dilutions of the antibody for 30 min at ambient temperature in culture medium prior to adding the test samples to the plates.

RESULTS

Purification and Characterization of Membrane-tethered Human Sonic hedgehog—When full-length human Shh was expressed in High Five insect cells, over 95% of the NH2-terminal fragment was in a cell-associated form. The Shh was purified from a detergent lysate of the cells by a combination of SP-Sepharose chromatography and immunoaffinity chromatography on a 5E1-Sepharose column. By SDS-PAGE, the purified protein migrated as a single sharp band with apparent mass of 20 kDa (Fig. 1, lane c). The protein migrated faster by about 0.5 kDa than a soluble version of the protein that had been produced in E. coli (Fig. 1, lanes b-d), consistent with previously published data (19). Similarly, as described (19), the soluble and membrane-bound Shh proteins were also readily distinguishable by reverse phase HPLC, where the tethered form eluted later in the acetonitrile gradient. The concentration of acetonitrile needed for elution of the membrane-bound form was 60% versus 45% for the soluble form, indicating a significant increase in the hydrophobicity of the protein.

ESI-MS data for the soluble and membrane-bound forms of Shh showed primary species with masses of 19,560 and 20,167 Da, respectively (Fig. 2). The measured mass of 19,560 Da matches the predicted mass for Shh starting with Cys-24 and terminating with Gly-197 (calculated mass of 19,560.02 Da). By contrast, the 20,167 Da mass did not match the predicted mass for residues 24–197 plus cholesterol (calculated mass of 19,928.64 Da), nor could the difference in the masses of the tethered and soluble forms, 607 Da, be accounted for by any known modification or by aberrant proteolytic processing. Since Porter et al. (18) had previously demonstrated that Drosophila hedgehog contained a cholesterol moiety, it was possible that the mass difference in the human Shh was due, at least in part, to cholesterol (calculated average mass for esterified cholesterol is 368.65 Da). Indeed, the presence of a minor component in the mass spectrum of tethered Shh at 19,796 Da (371 Da smaller than the primary ion) supported this notion.

Further evidence for cholesterol modification was obtained by treating the tethered Shh with a mild alkali under conditions that can hydrolyze the cholesterol linkage without disrupting peptide bonds (18), and then reanalyzing the reaction products by MS. Base treatment caused a 387-Da shift in the observed mass of the tethered Shh, which is consistent with the loss of cholesterol plus water (see Table I), while the mass of soluble Shh was not affected by the treatment. Together, these observations suggested that the membrane-tethered human Shh contained two modifications with total mass of 607 Da, a cholesterol (368.65 Da), and a second moiety with mass of 238 Da. The similarity in mass between this value and the mass of a palmitoyl group (calculated mass of 238.47 Da) suggested that the protein might be palmitoylated, which was verified through more rigorous biochemical studies (see below). While the mass data clearly support the notion that Shh is cholesterol modified and have extended this observation to human Shh, the loss of water following base treatment is inconsistent with the proposed model in which the cholesterol is attached to the COOH-terminal carboxyl group through an ester linkage (18, 19). Base hydrolysis of an ester linkage would not result in the loss of water. More extensive studies are needed to address this issue.
The distinctive chromatographic properties of the lipid-modified forms of Shh on reverse phase HPLC was used as an assay to quantify the extent of modification (see Fig. 3B). In this assay, the unmodified Shh elutes first (peak 1), followed by cholesterol-modified Shh (peak 2), and finally the Shh containing both the cholesterol and palmitic acid modifications (peak 3). Over 80% of the tethered Shh recovered from High Five cells contained both cholesterol and the palmitoyl moiety. The shoulder on peak 3 was caused by a modified form of the palmitoyl moiety, containing an unsaturated bond, that was identified through peptide mapping and sequence analysis by MALDI PSD measurement (data not shown). The mass of this variant was 2 Da smaller than that of the main peak.

The Membrane-tethered Form of Human Shh Contains a Palmitoyl Moiety—Direct evidence for palmitic acid modification was obtained using an in vitro modification reaction in which Shh was treated with [3H]palmitic acid under conditions that promote protein palmitoylation. As shown in Fig. 1 (lane e), Shh is readily labeled with the radioactive tracer. None of the approximately 100 other proteins in the reaction mixture were labeled (see the corresponding Coomassie Blue-stained gel profile in lane j), indicating a high degree of specificity of the palmitoylation reaction. As further evidence for the specificity of the palmitoylation reaction, we tested two Shh variants in which the site of palmitoylation (Cys-24, see below) had been eliminated. Fig. 1, lane f, shows results from the analysis of a truncated form of soluble Shh that was lacking amino acid residues 24–33 and lane g, of a mutant form of Shh containing a single Cys-24 to Ser point mutation. Neither of the variants was labeled.

Shh was also labeled with [3H]palmitic acid when High Five cells that had been infected with baculovirus encoding the full-length gene for Shh were analyzed (data not shown). In this study, lysates from the [3H]palmitic acid-labeled cells were treated with 5E1-Sepharose or resin from a control mAb of the same isotype, and the immunoprecipitates subjected to SDS-PAGE and fluorography. The mature Shh band was readily detected in the 5E1-treated sample and absent from the control sample. No label was detected in 5E1 immunoprecipitates from a control lysate from [3H]palmitic acid-labeled cells that had been infected with a non-hedgehog encoding virus stock.

Localization of the Palmitoyl Group within the Human Shh Sequence—The site of palmitoylation within the human sequence was identified by using a combination of peptide mapping and amino acid sequence analysis. Fig. 4B shows results from a peptide mapping analysis of the soluble protein with an LC-MS readout. Mass data accounting for over 98% of the Shh sequence could be accounted for from a peptide mapping study. The peak noted with an asterisk corresponds to the NH₂-terminal peptide (residues 1–9 plus a pyridylethyl group, observed mass 983.50 Da, calculated monoisotopic mass 983.49 Da; Table I). In the corresponding analysis of the tethered product (Fig. 4A), this peptide was missing and instead a more hydrophobic peptide with a mass of 1221.79 Da was observed (noted with an asterisk). The 1221.79 Da moiety is consistent with the

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**TABLE I**

**Characterization of tethered human Shh by MS**

| Protein                        | Mass (Da)   |
|--------------------------------|-------------|
| KOH-treated Shh                |             |
| No tether (− treatment)        | 19,560.02   |
| Tethered (+ treatment)         | 20,167.14   |
| NH₂-terminal endoproteinase    |             |
| Lys-C peptide (MH⁺)            |             |
| No tether                      | 983.49      |
| Tethered                       | 1,221.72    |

*All mass values presented in the paper for peptides are for their protonated masses.

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**FIG. 3. Analysis of tethered Shh by reverse phase HPLC.** Soluble human Shh (A), tethered human Shh from High Five insect cells (B), and EBNA-293 cells (C), and cell-associated rat Shh (D) were subjected to reverse phase HPLC on a narrow bore Vydac C₄ column (2.1-mm inner diameter × 250 mm). The column was developed with a 35 min 0–80% acetonitrile gradient in 0.1% trifluoroacetic acid at 0.25 ml/min and the effluent monitored using a photodiode array detector from 200 to 300 nm (data shown at 214 nm). Peak fractions were collected and characterized further by SDS-PAGE and MS (data summarized in Table II).
presence of a modified form of the NH₂-terminal peptide, i.e. 983.49 Da for the pyridylethylated peptide plus 238.23 Da. The 1221.79 Da peptide was next subjected to sequence analysis by MALDI PSD measurement. The resulting PSD spectrum is shown in Fig. 5. Ions corresponding to b₁, b₂, b₄, b₅, b₆ + H₂O, y₈, y₇, y₅, y₄, y₃, y₂, and y₁ fragments were detected, which confirmed the sequence. All of the b ions contained the added 238.23 Da mass adduct, indicating that the adduct was on Cys-24.

Since cysteine is the usual site for protein palmitoylation in vivo, it was not surprising to find the adduct attached to Cys-24. However, three pieces of evidence suggested that the lipid was attached to the α-amino group on the cysteine and not to the thiol. First, in the peptide mapping study, we used 4-vinyl pyridine as a spectroscopic tag to monitor free thiol groups (28). Pyridylethylation is highly specific for Cys thiols and adds a 105.06 Da (monoisotopic mass) unit that can be detected by MS. In the peptide map of tethered Shh, as expected, we observed three Cys-containing peptides based on the characteristic absorbance maxima of the pyridylethyl group at 254 nm and, by mass, these peptides contained the 105 Da addition characteristic of the pyridylethyl moiety. The peptides containing Cys-102 and Cys-183 were only 105 Da larger than the predicted mass of the peptide sequence alone. In contrast, the Cys-24 containing peptide was 343 Da larger, consistent with the presence of both the palmitoyl and pyridylethyl moieties. Since the observed Cys-24 containing fragments seen in the PSD spectrum contained both the palmitoyl and pyridylethyl moieties, the palmitoyl group must be on the α-amino group. In support of this conclusion, ions at m/z of 308.48 and 676.86 were observed that correspond to b₁ and b₅, but that have lost the 2-4-pyridylthiethiol group (Fig. 5). Second, when the tethered Shh was subjected to automated NH₂-terminal Edman sequencing, no sequence was obtained, suggesting blockage of the NH₂-terminal α-amino. The predicted sequence was observed when the corresponding pyridylethylated form of soluble human Shh (residues 24–197) was subjected to NH₂-terminal sequencing. Third, when tethered Shh was subjected to base treatment (Table I), the cholesterol moiety was released from the protein, while the palmitoyl group was resistant to the treatment. If the palmitoyl group had been attached to the thiol, base treatment would have also caused the release of the palmitoyl adduct. As further evidence for the N-palmitoylation modification, we chemically modified Shh with palmitic acid added at its NH₂ terminus and then subjected this product to the same battery of tests that had been used to characterize the biosynthetically modified Shh. The data were indistinguishable except that only the fully saturated form of the palmitoylated product was detected.

**Lipid Modification Results in Enhanced Potency in the C3H10T1/2 Assay**—The mouse embryonic fibroblast line C3H10T1/2 is a mesenchymal stem cell line that, under defined conditions, can differentiate into adipocytes, chondrocytes, and bone osteoblasts (29, 30). Bone morphogenetic proteins drive the differentiation of C3H10T1/2 cells into the bone cell lineage and alkaline phosphatase induction has been used as a marker for this process (30). Shh has a similar effect on C3H10T1/2 cells (26) and we routinely use the alkaline phosphatase induction by Shh as a quantitative measure of its in vitro potency. Fig. 6A shows results from a study in which tethered Shh was tested for function in the C3H10T1/2 cell assay. In this assay, soluble Shh produces a dose-dependent response with an IC₅₀ of 1 μg/ml and a maximal signal at 3 μg/ml. Tethered Shh produced a similar dose-dependent response in the assay but with an IC₅₀ of 0.03 μg/ml and a maximal signal at 0.1 μg/ml, indicating that it was about 30 times as potent as soluble Shh in the C3H10T1/2 assay. To verify that the observed activity was hedgehog specific, we tested whether the activity could be neutralized with the anti-hedgehog neutralizing mAb 5E1. Both soluble and tethered Shh were inhibited by 5E1 treatment (Fig. 6B). Inhibition of the tethered Shh required one-tenth as much 5E1 consistent with its increased specific activity in the assay.

Tethered Shh was also tested in a receptor binding assay, monitoring its ability to bind patched-1, using a modified version of a recently published assay (9). The tethered Shh showed dose-dependent binding to patched-1 expressing cells with an apparent IC₅₀ of 400 ng/ml (Fig. 7). In the same assay, soluble Shh bound to patched-1 with an apparent IC₅₀ of 150 ng/ml, indicating that the tethered form was not significantly different from soluble Shh for binding to patched-1.

**Characterization of Membrane-tethered Human Shh from EBNA-293 Cells**—In order to assess whether palmitoylation

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2 D. P. Baker, C. Zeng, and D. Wen, unpublished observations.
was a general modification pathway for Shh or whether it was specific to insect cell-expressed hedgehog, the protein was also produced in a mammalian system in EBNA-293 cells. The tethered Shh was purified from EBNA-293 cells and characterized exactly as described for the insect cell-derived product. As with the insect cell-derived product, the electrophoretic mobility of the tethered Shh from EBNA-293 cells was slightly faster than soluble Shh, it was retarded similarly on the C<sub>4</sub> column in the reverse phase HPLC analysis and, by mass spectrometry, it contained an ion corresponding to the protein with both palmitic acid and cholesterol modifications. However, unlike the insect cell-derived product where over 80% of the protein contained both the palmitoyl and cholesterol moieties, the HPLC elution profile and data from mass spectrometry revealed that only about 30% of the mammalian cell-derived protein contained both modifications (see Table II and Fig. 3 C). Interestingly, the insect cell and mammalian cell-derived products showed comparable activity in the C3H10T1/2 assay, suggesting that Shh modified with cholesterol only, or with cholesterol and palmitic acid are equally functional.

A second difference between the two expression systems was that a greater proportion of the Shh from EBNA-293 cells was not membrane-associated. Whereas this fraction accounted for less than 5% of the Shh out of High Five cells, as much as 20% of the hedgehog from the EBNA-293 cells was in a soluble form. Although we have not characterized the soluble product, we infer from this observation that the double lipid tether is a more stringent method of membrane attachment than the cholesterol alone and therefore might better account for the short range-long range signaling property of hedgehog proteins. Whether the second lipid attachment site is used simply to further stabilize the association of the protein with membrane, or whether it plays a more active role and affects hedgehog conformation or hedgehog receptor/ligand contacts remains to be determined.

Lipid-modified Rat Sonic hedgehog—When the NH<sub>2</sub>-terminal fragment of rat Shh (residues 25–198 of the full-length protein) was expressed in insect cells, the majority of the protein was secreted into the culture medium since the construct lacked the autoprocessing domain responsible for attaching cholesterol to the COOH terminus. When purified, this soluble form had a similar specific activity (as measured in the C3H10T1/2 assay) as the soluble, NH<sub>2</sub>-terminal fragment of human Shh expressed and purified from E. coli. However, a small fraction of the product (<1% of the total hedgehog protein as determined by Western blot analysis) had significantly higher specific activity in the C3H10T1/2 assay. This form was found to be associated with membrane fragments (presumably liberated into the culture medium after baculovirus-induced lysis of the infected cells) and could be separated from the soluble form by size exclusion chromatography.

Subsequently, we determined that the same high activity form could be purified more readily from the infected cells rather than from the culture medium. This cell-associated form was purified in the presence of detergent on SP-Sepharose resin and on 5E1-Sepharose, and was characterized by using the same strategies described for the tethered form of human Shh. The cell-associated rat Shh had a specific activity in the C3H10T1/2 assay that was very similar to the tethered form of human Shh. However, unlike the tethered form of human Shh, which was found to have only a palmitoyl group attached to the NH<sub>2</sub>-terminal cysteine (either in the absence or presence of cholesterol at the COOH terminus), ions consistent with myristoylated, palmitoylated, stearoylated, and arachidoylated forms of the rat protein were detected (see Fig. 3 D and Table II). Furthermore, as expected, there was no evidence for cholesterol in the rat Shh. When the sample was subjected to alkylation by 4-vinyl pyridine and endoproteinase Lys-C peptide mapping, and the profile compared with the digest of soluble human Shh, six new, late-eluting, peaks were detected that were characterized directly by mass spectrometry and then by MALDI PSD sequencing. Each peak corresponded to a different lipid-linked variant of the NH<sub>2</sub>-terminal peptide and represented myristic, palmitic, and stearic acid modifications, and their respective unsaturated forms which differed in mass by 2 Da. The arachidic acid-modified NH<sub>2</sub>-terminal peptide was not detected presumably because it remained bound to the HPLC column.

DISCUSSION

We have demonstrated that human Sonic hedgehog is tethered to cell membranes in two forms: one form contains cholesterol and is therefore analogous to the form previously reported for Drosophila hedgehog (18), and a second, novel, form that contains both a cholesterol and a palmitoyl group. Although naturally occurring Shh has not been purified and character-
Palmitic Acid-modified Form of Human Sonic hedgehog

Fig. 6. Increased activity of tethered human Shh in the C3H10T1/2 assay. The relative potencies of soluble and tethered Shh alone (A) or in the presence of the anti-hedgehog neutralizing mAb 5E1 (B) were assessed on C3H10T1/2 cells measuring alkaline phosphatase induction. The numbers presented reflect the averages of duplicate determinations. A, serial 2-fold dilutions of soluble ○ and tethered ▼ Shh were incubated with the cells for 5 days and the resulting levels of alkaline phosphatase activity measured at 405 nm using the AP chromogenic substrate p-nitrophenyl phosphate. B, serial 2-fold dilutions of the mAb 5E1 were incubated with Shh (5 μg/ml, black bars) or tethered Shh (0.25 μg/ml, gray bars) or vehicle control without Shh added (white bar) for 30 min and then subjected to analysis in the C3H10T1/2 assay.

Fig. 7. Analysis of human Shh in a receptor binding assay. The relative in vitro potency of soluble ○ and tethered ▼ Shh for binding to patched-1 was assessed on patched-1 transfected EBNA-293 cells by FACS analysis. Percent binding of Shh was measured by the ability of the samples to compete with Shh-Ig for binding to the cells. Serial dilutions of the test samples plus 5 nM Shh-Ig were incubated with the EBNA-293 cells. After the cells were washed, bound Shh-Ig was quantified by mean fluorescence using a phycoerythrin-labeled donkey anti-human IgG1 (Jackson ImmunoResearch) probe as the readout. The data were fitted to a hyperbolic curve by non-linear regression.

Table II

| Protein | Mass (Da) | Calculated | Measured |
|---------|-----------|------------|----------|
| a. Bacterial expressed (no tether) | 19,560.02 | 19,560 |
| b. Baculovirus expressed (tethered) + Palmitoyl- | 19,798.49 | 19,796 |
| + Palmitoyl-cholesterol- | 20,167.14 | 20,168 |
| c. EBNA-293 cell expressed (tethered) Peak 1 (9% of total hedgehog) | 19,560.02 | 19,581 |
| No tether | 18,700.02 | 18,712 |
| No tether (des-1–9) | 18,912.48 | 18,889 |
| Peak 2 (61% of total hedgehog) + Cholesterol- | 19,928.64 | 19,934 |
| + Cholesterol- (des-1–10) | 18,912.48 | 18,889 |
| Peak 3 (30% of total hedgehog) + Palmitoyl-cholesterol- | 20,167.14 | 20,174 |
| d. Lipid-modified rat Shh Unmodified | 19,632.08 | 19,632 |
| Myristoyl- | 19,870.55 | 19,868 |
| + Palmitoyl- | 19,870.55 | 19,868 |
| + Stearoyl- | 19,898.60 | 19,896 |
| + Arachidoyl- | 19,926.66 | 19,925 |

The presence of the palmitoyl moiety both in the insect cell and mammalian cell-derived human Shh preparations suggests that the modification is likely to be physiologically relevant. The site of the palmitic acid modification was identified through a combination of peptide mapping and sequence analysis to be the NH2 terminus of the protein, on Cys-24. Both tethered forms were equally active in the C3H10T1/2 alkaline phosphatase assay and are about 30 times more potent than the soluble human Shh lacking the tether(s). The lipid modifications, however, did not significantly affect the apparent binding affinity of Shh for the hedgehog receptor patched-1. patched-1 is not highly expressed on C3H10T1/2 cells, but is induced following Shh treatment (data not shown), raising the possibility that patched-2 or other hedgehog-binding proteins might contribute to hedgehog activity on this cell line. While the basis for the increase in activity of the tethered Shh in the C3H10T1/2 assay is unknown, the data clearly indicate that membrane presentation is an important component of the protein’s function.

While lipid attachment via glycosylphosphatidylinositol linkages provides a mechanism for increasing the lateral movement of a protein in the membrane, the role of the cholesterol and cholesterol/palmitic acid modifications on Shh remains to be defined. The ability to purify both tethered forms of Shh in a functional state should allow for more rigorous mechanistic studies into the functional significance of these modifications. In particular, although it is well established that Shh participates in both short range, contact-dependent, and long range, diffusible signaling, the nature of the molecular forms underlying these two activities is unclear. Both activities reside in the NH2-terminal 20-kDa portion of the protein (2, 17), and it has been proposed that the cholesterol-modified protein, which is membrane-associated and therefore presumably localized, is responsible for short range signaling, while the long range signal may be due to limited diffusion of the cholesterol form, or due to the enzymatic liberation of the soluble (unmodified) form from the membrane (19). The additional lipid modifications described in the present work may offer an additional means of...
regulating the short/long range signaling activities of hedgehog proteins, with the doubly modified form, for example, providing the localized, contact-dependent signal, and the singly modified, or unmodified, form supplying the long range signal. Our observation that a substantially larger proportion of the EBNA-293 cell-derived Shh was in a soluble form supports this possibility.

Protein palmitoylation is a common post-translational modification that occurs late in the processes leading to maturation (31, 32). The process is dynamic and involves separate enzymes that add and remove the modification. The most common functions of palmitoylation are to target a protein to its site of action, to promote protein-protein interactions, and to mediate protein-membrane interactions (33). While the difference in the extent of palmitoylation in the insect and mammalian cell-derived preparations (80% in insect cells versus 30% in EBNA-293 cells) was surprising, we do not know if this is biologically significant or whether it simply reflects differences in the cellular machinery of the two host systems for adding and removing the palmitoyl moiety. Most, if not all, palmitoylated proteins are intracellular, or are transmembrane proteins with the palmitoyl group attached to the cytoplasmic domain. The observation that an extracellular protein such as Shh is palmitoylated is highly unusual and further adds to the complex nature of this protein. To assess whether the palmitoylation reaction was unique to human Shh, or whether it might occur on other hedgehog proteins, we tested whether human Ihh also could be palmitoylated. The human Ihh was also modified (Fig. 1, lane h), indicating that palmitoylation is likely to be a common feature of hedgehog proteins.

The ability to label Shh directly with radioactive palmitic acid in a cell-free system provided a simple screen for the amino acid residues required for modification. The role of Cys-24 is highlighted by the observation that wild type soluble Shh is readily labeled while the Ser-24 mutant is not. The inability to label the Ser-24 mutant argues against a simple reaction mechanism where the palmitoyl group is attached directly to the NH2 terminus, since under the test conditions used the serine should have substituted for the cysteine. We also tested the role of the free NH2 terminus by using a form of Shh that is responsible for the enhanced activity. Reconstitution experiments into positively and negatively charged liposomes by detergent dilution over a wide range of lipid:protein ratios (w/w) from 1:1 to 100:1 had no affect on tethered Shh activity in the C3H10T1/2 assay (data not shown). The fact that tethered Shh is more efficacious than the soluble form suggests that it would be useful to develop better defined multivalent forms of the protein.

The mechanism of action of hedgehog proteins is unknown. The crystal structure of the signaling domain of Shh revealed a tetrahedrally coordinated zinc ion that was structurally analogous to that found in zinc hydrolases (35). Although this is suggestive of an enzymatic role, no substrate has yet been identified. The recent identification of patched-1 as a receptor for hedgehog has provided a more defined focus for future research efforts (9). Detailed structure-function studies suggest that the NH2-terminal region of Shh is important for activity in the C3H10T1/2 assay and therefore it was not surprising that palmitoylation of the NH2 terminus affected function. In these studies, mutations that resulted in the replacement of Cys-24 with serine, moved the Cys from its natural position in the sequence, or added additional amino acid residues at the NH2 terminus resulted in loss of activity in the C3H10T1/2 assay.

Little is known about the structure of this region of the protein, since the crystal structure of Shh lacks the first nine amino acids (35). The fact that both ends of the protein become lipid modified suggests that they are either close by or at least oriented on the same face of the protein. During the purification of soluble forms of Shh, we observed that the NH2-terminal modification alone is as effective as the cholesterol modification at enhancing the activity of Shh. Thus, if the cholesterol tether were released, the protein would remain in its high activity state. Second, the high degree of specificity for palmitate in the human cholesterol-modified product and apparent lack of specificity in the modification of the rat soluble protein suggests that the proteins were subjected to different membrane compartments at some point during their synthesis. A better understanding of this phenomenon may assist in understanding the significance of the cholesterol modification, since hedgehog is the only known cholesterol-modified protein. Third, since many laboratories use soluble forms of hedgehog proteins to study structure-function relationships, it is possible that certain activities that have been attributed to the soluble form may in fact be due to trace amounts of an acylated product present in the preparation or which forms during the experiment.

The strategy used for purifying tethered Shh was based on previous experience with glycosylphosphatidylinositol-linked LFA3 (34). With the glycosylphosphatidylinositol-linked protein, detergent dilution resulted in the formation of a soluble octomeric LFA3 complex that was about 500 times as active as a soluble form of the protein. Unlike glycosylphosphatidylinositol-LFA3, detergent dilution of the tethered Shh produced a high molecular weight complex of undefined structure. Since the cell-based assay employed involves such a long incubation time (3–5 days), we do not know the actual state of the protein that is responsible for the enhanced activity. Reconstitution experiments into positively and negatively charged liposomes by detergent dilution over a wide range of lipid:protein ratios (w/w) from 1:1 to 100:1 had no affect on tethered Shh activity in the C3H10T1/2 assay (data not shown). The fact that tethered Shh is more efficacious than the soluble form suggests that it would be useful to develop better defined multivalent forms of the protein.

Interactions of the Shh N-terminal region with the patched receptor are as yet undetermined. Since the N-terminal region is highly susceptible to proteolysis with cleavages occurring most frequently after the basic residues, Arg-28, Lys-32, and Arg-33. By contrast, the tethered, palmitoylated form was less proteolyzed. Since changes in protease suscepti-

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bility are often suggestive of conformational differences in a protein, it will be interesting to determine if palmitoylation impacts hedgehog structure.

The role of hedgehog proteins as fundamental growth/differentiation signals in embryonic development has raised interest in their potential use as therapeutic products. While significant advances have been made assessing function with soluble forms of Shh, little is known about the more natural tethered forms. We have purified and characterized human and rat forms of Shh, and demonstrated that the lipid-modified protein can be isolated in a functional state. Our studies indicate that the tethered modified protein, it will be interesting to determine if palmitoylation impacts hedgehog structure.

Acknowledgments—We thank Stephan Miller and Weihong Shen for their contributions to this work and Michele Sanicola, Jeff Porter, Nagesh Mahanthappa, and Tom Ingolia for helpful discussion and for critical reading of the manuscript.

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