Enhanced Potency of Human Sonic Hedgehog by Hydrophobic Modification

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ABSTRACT: Post-translational modifications of the developmental signaling protein Sonic hedgehog (Shh) by a long-chain fatty acid at the N-terminus and cholesterol at the C-terminus greatly activate the protein in a cell-based signaling assay. To investigate the structural determinants of this activation phenomenon, hydrophobic and hydrophilic moieties have been introduced by chemical and mutagenic methods to the soluble N-terminal signaling domain of Shh and tested in both in vitro and in vivo assays. A wide variety of hydrophobic modifications increased the potency of Shh when added at the N-terminus of the protein, ranging from long-chain fatty acids to hydrophobic amino acids, with EC_{50} values from 99 nM for the unmodified protein to 0.6 nM for the myristoylated form. The N-myristoylated Shh was as active as the natural form having both N- and C-terminal modifications. The degree of activation appears to correlate with the hydrophobicity of the modification rather than any specific chemical feature of the adduct; moreover, substitution with hydrophilic moieties decreased activity. Hydrophobic modifications at the C-terminus of Shh resulted in only a 2−3-fold increase in activity, and no activation was found with hydrophobic modification at other surface positions. The N-terminal modifications did not appear to alter the binding affinity of the Shh protein for the transfected receptor protein, Patched, and had no apparent effect on structure as measured by circular dichroism, thermal denaturation, and size determination. Activation of Desert Hh through modification of its N-terminus was also observed, suggesting that this is a common feature of Hh proteins.

Hedgehog (Hh)[1] proteins are a family of extracellular signaling molecules involved in patterning events in many developmental pathways (*1*). Hh proteins are expressed on the surface of a restricted set of cells, localizing their sites of action. Members of this family have been identified in both vertebrates and invertebrates, and three Hh's, referred to as Sonic, Indian, and Desert, with different tissue distributions, have been identified in rodents and humans. Three cell surface proteins that bind Hh have been described: Patched (Ptc) [*2*−*5*; reviewed by Ingham (*6*)], Patched 2 (*7*−*9*), and Hedgehog interacting protein (*10*). Ptc is a multipass membrane-spanning protein with homology to transporter or channel proteins (*11*, *12*), and it has a region that is homologous to the cholesterol binding domain of the cholesterol-regulated proteins 3-hydroxy-3-methylglutaryl-CoA reductase and SREBP (*13*, *14*). The initial model of signaling was that Ptc formed an inhibitory complex with Smoothened, a seven-transmembrane-spanning protein, similar to G-protein-coupled receptors. Hh signaling occurred when the binding of Hh to Ptc relieved the negative regulation of Smoothened (*3*−*6*). However, there have also been suggestions that the signaling mechanism is fairly complex and includes trafficking events within the cell (*15*). For example, signaling is disrupted by steroidal-like compounds that can also interfere with the intracellular trafficking of cholesterol (*16*, *17*) and the major fraction of Ptc appears to be in intracellular vesicles and not on the cell surface (*18*, *19*). Recently, evidence for a different model has been presented in which Ptc changes the phosphorylation state of Smoothened and thereby prevents its accumulation at the cell surface. In this model, Hh relieves the Ptc effect on Smoothened by inducing the internalization of Ptc (*20*).

The Hh genes encode proteins with masses of ∼45 kDa consisting of a 20 kDa N-terminal signaling domain and a 25 kDa C-terminal autocatalytic processing domain (*21*). Beachy and co-workers have shown that Shh undergoes a unique post-translational processing event in which autoproteolytic cleavage catalyzed by the C-terminal domain releases the N-terminal domain with the concomitant introduction of cholesterol at its new C-terminus (*22*). More recently, we have shown that when Shh is expressed in insect or EBNA 293 cells a high percentage of the protein has, in addition to the C-terminal cholesterol, a long-chain fatty acyl group in an amide linkage to the N-terminal cysteine (*23*). The process leading to fatty acyl modification also appears to be coupled with proteolytic maturation; however, the mechanism for this is unknown.

When the lipid-linked form of the protein is expressed in cell culture, it remains cell-associated (*24*, *25*). In contrast, when the N-terminal signaling domain is expressed without

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[1] Abbreviations: Hh, hedgehog; Shh, Sonic hedgehog; Ptc, Patched; DTT, dithiothreitol; CoA, coenzyme A; PSD, post-source decay; ESI-MS, electrospray-ionization mass spectroscopy; PTH, phenylthiohydantoin; CD, circular dichroism; HPLC, high-performance liquid chromatography.

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the C-terminal processing domain, most of the protein is not lipid-modified, and it is released into the cell supernatant (*26*). This difference in cell association also appears to occur when expression of the full-length and N-terminal domain are compared in vivo, leading to the conclusion that one or both of the lipid adducts are responsible for tethering Hh to the cell surface (*25, 27*). Recently, it has been reported that another protein, Dispatched, also with a sterol-sensing domain, participates in release of lipid-modified Hh for signaling to adjacent cells (*28*). It has been reported that the lipid modifications target Hh to lipid microdomains of the plasma membrane, referred to as rafts, that have a specialized function in polarized intracellular sorting and signal transduction (*29*). Longer range signaling by Hh proteins has also been reported in some developmental systems (*30*−*32*), in which different concentrations of Hh induce different cell fates. Whether this action of Hh at a distance is due to the release of Hh from cells, to the movement of Hh by other transport mechanisms, or to secondary effects is currently under investigation (*33*−*35*).

An intriguing feature of the lipid-modified Hh proteins purified from the insect and EBNA 293 cell lines is that they were found to be much more active than the unmodified protein in an in vitro, cell-based signaling assay that measures the induction of alkaline phosphatase activity in C3H10T1/2 cells (*23*). Here we report further investigation of the types and locations of hydrophobic adducts that increase Hh activity in this assay and in some in vivo settings. We find that long-chain fatty acid amides at the N-terminus are the most activating, resulting in over 100-fold increases in potency. However, a remarkable range of other hydrophobic modifications can also activate the protein, including the addition of hydrophobic amino acids in place of the N-terminal cysteine. These modifications do not alter the apparent binding affinity of the Hh protein to its receptor, Ptc, raising the question as to how the modifications modulate activity. The modified forms provide novel tools for probing the biology and mechanism of action of the Hh proteins.

EXPERIMENTAL PROCEDURES

Bacterial Expression and Purification of the Human Shh N-Terminal Domain. The N-terminal signaling domain of human Shh (corresponding to residues 24−197) was produced in an *E. coli* expression system as a six histidine-tagged fusion protein with an enterokinase cleavage site engineered into the construct immediately adjacent to the N-terminal cysteine (Cys-24) of the mature wild-type Shh sequence. The expression vector, designated p6H-Shh, was provided by Dr. David Bumcrot (Curis, Inc.). The expression, purification, and removal of the histidine-tag from the N-terminal domain are described in Williams et al. (*36*). This form of the protein will be referred to as unmodified Shh.

Construction of huShh C24 Mutants. A 584 bp *Nco*I−*Xho*I restriction fragment carrying the histidine-tagged Shh N-terminal domain from p6H-Shh was subcloned into the pUC-derived cloning vector pNN05 to construct the plasmid pEAG649. C24 mutants of soluble Shh were made by unique site elimination mutagenesis of the pEAG649 plasmid template using a Pharmacia kit following the manufacturer's recommended protocol. In designing the mutagenic primers, if a desired mutation did not produce a restriction site change, a silent mutation producing a restriction site change was introduced into an adjacent codon to facilitate identification of mutant clones following mutagenesis. Mutations were confirmed by DNA sequencing through a 180 bp *Nco*I−*Bgl*II restriction fragment carrying the mutant Shh proteins' N-termini. Expression vectors were constructed by subcloning each mutant plasmid's 180 bp *Nco*I−*Bgl*II fragment and the 404 bp *Bgl*II−*Xho*I fragment from pEAG649 into the phosphatase-treated 5.64 kb *Xho*I−*Nco*I pET11d vector backbone of p6H-Shh. The presence of the introduced restriction site change was reconfirmed in the expression vector for each C24 mutant. Expression vectors were transformed into competent *E. coli* BL21(DE3)pLysS cells (Stratagene) (*37*).

Purification of Cys-24 Mutants of Shh. The histidine-tagged mutant Shh proteins were purified from the bacterial pellets as described for the unmodified Shh protein (*36*) except, in some cases, for two modifications: (1) The Phenyl Sepharose step was eliminated, and instead the protein pool from the first Ni-NTA agarose column was dialyzed into 25 mM Na_2HPO_4, pH 8, 400 mM NaCl, 0.5 mM dithiothreitol (DTT) in preparation for the enterokinase cleavage step. (2) The final ion exchange step was changed from step elution on SP-Sepharose Fast Flow to gradient elution from a CM-Poros column (4.6 × 100 mm, PerSeptive Biosystems). The pooled fractions from the second Ni-NTA agarose column, containing from 3 to 15 mg of protein from 1 L of bacterial culture, were diluted 10-fold into the CM column running buffer (50 mM NaH_2PO_4, pH 6.0) and eluted with a 0−800 mM NaCl gradient over 30 column volumes. The pooled peak fractions from this step were dialyzed into 5 mM NaH_2PO_4, pH 5.5, 150 mM NaCl and were stored at −70 °C. Mass spectrometry of the purified proteins gave the predicted mass ions for each purified form (data not shown).

Fatty Acid Acylation with CoA Esters. Palmitoylated, myristoylated, lauroylated, decanoylated, and octanoylated Shh proteins, carrying a single acyl chain attached to the α-amine of the N-terminal cysteine, were produced in reaction mixtures containing 41 μM unmodified Shh, 40 mM Na_2HPO_4, pH 7.0, and either 410 μM (10-fold molar excess) palmitoyl-CoA, myristoyl-CoA, or lauroyl-CoA plus 25 mM DTT, or 4.1 mM (100-fold molar excess) decanoyl-CoA or octanoyl-CoA plus 0.5 mM DTT. The fatty acid CoA esters were obtained from Sigma. Reaction mixtures were incubated at 28 °C for 24 h. The reaction products at this stage contained fatty acid moieties attached at both the α-amine and thiol group of the N-terminal cysteine. The thioester-linked acyl group was selectively removed by adding 0.11 volume of 1 M Na_2HPO_4, pH 9.0, and 0.11 volume of 1 M hydroxylamine followed by incubation at 28 °C for 18 h in the presence of 25 mM DTT. Octyl-β-D-glucopyranoside (0.25 volume of 5%) was then added and the mixture incubated for 1 h at room temperature. The lipid-modified proteins were purified from the unmodified protein by chromatography on a Bioscale S column (BioRad) in the presence of 1% octylglucoside. The purified proteins were dialyzed against 5 mM NaH_2PO_4, pH 5.5, 150 mM NaCl, 1% octyl-β-D-glucopyranoside, 0.5 mM DTT, and were stored at −70 °C. Octyl-β-D-glucopyranoside was required to maintain solubility of the palmitoylated protein; the lower chain length forms remained soluble when detergent was

removed by dialysis. Electrospray-ionization mass spectroscopy (ESI-MS) data confirmed a single site of modification (data not shown). The site of modification was determined by peptide mapping and sequencing (see Results).

Modification of Shh with Formaldehyde To Form an N-Terminal Thiaproline Derivative. Unmodified Shh at 3 mg/mL (153 μM) in 5 mM NaH_2PO_4, pH 5.5, 150 mM NaCl, 0.5 mM DTT was treated with 0.1% formaldehyde at room temperature for 1 h and the modified protein immediately purified on a CM-Poros column as described above. ESI-MS data are consistent with modification of Shh with a single formaldehyde moiety to form the thiaproline analogue, and the thiaproline amino acid was identified in the first cycle of N-terminal sequencing (data not shown). The site of modification was determined by peptide mapping and sequencing (see Results).

Modification of Shh with Thiol-Specific Maleimide and Iodoacetyl Reagents. Unmodified Shh at 3 mg/mL (153 μM) in 5 mM NaH_2PO_4, pH 5.5 or pH 7.0 (for *N*-isopropyliodoacetamide), 150 mM NaCl, 0.5 mM DTT was treated with either 10 mM *N*-ethylmaleimide (Sigma), 300 μM *N*-octylmaleimide (gift from Jeff Porter, Curis, Inc.), 10 mM iodoacetic acid (Sigma), or 1 mM *N*-isopropyliodoacetamide (Molecular Probes) at 4 °C and then dialyzed into 5 mM NaH_2PO_4, pH 5.5, 150 mM NaCl. The products were purified on a Poros CM column and dialyzed against 5 mM NaH_2PO_4, pH 5.5, 150 mM NaCl, 0.5 mM DTT. ESI-MS data confirmed a single site of modification (data not shown). Modification of cysteine mutants of Shh with *N*-(1-pyrenyl)-maleimide (Sigma), β-(4-hydroxyphenyl)ethylmaleimide (Pierce), *N*-(1-naphthyl)maleimide (Aldrich), and *N*-(1-octyl)-maleimide was carried out as described previously (*38*).

Peptide Mapping. Sample containing about 20 μg of protein in 50 μL of 6 M guanidine hydrochloride, 0.1 M Tris-HCl, pH 8.0, 1 mM EDTA, 0.5 mM DTT was treated with 0.5 μL of 4-vinylpyridine (Sigma, final concentration 100 mM) for 1.5 h at room temperature. The *S*-pyridylethylated protein was precipitated by addition of 40 volumes of ice-cold ethanol. The solution was stored at −20 °C for 1 h and then centrifuged at 14000*g* for 6 min at 4 °C. The supernatant was discarded, and the precipitate was washed once with ice-cold ethanol. The protein pellet was stored at −20 °C. The alkylated protein was suspended at 0.4 mg/mL in 1 M guanidine hydrochloride, 0.5 M Tris-HCl, pH 6.0, and digested with endoproteinase Lys-C from Achromobacter (Wako Pure Chemical Industries, Ltd.) at a 1:10 enzyme:substrate ratio. The digestion was conducted at room temperature for 20−24 h and then stored at −70 °C. Digests were analyzed by reversed-phase HPLC using a Waters 2690 Alliance Separations Module with a model 996 photodiode array detector (Waters Corp., Milford, MA). The column used was either a 2.0 mm internal diameter × 25 cm YMC C_{18} column (AA12S052502WT) or a 2.1 mm × 25 cm Vydac C_{18} column (218TP52). The running conditions were a 100 min gradient (0−80% acetonitrile) in 0.1% trifluoroacetic acid at a flow rate of 0.2 mL/min. Individual peaks were collected for further analysis.

Mass Determination. The molecular masses of intact proteins were determined by ESI-MS on a Micromass Quattro II triple quadrupole mass spectrometer. Samples were desalted using an on-line Michrom Ultrafast Microprotein Analyzer system with a Reliasil C_4 (1 mm internal diameter × 50 mm) column. The flow rate was 50 μL/min. All electrospray mass spectral data were processed using the Micromass MassLynx data system. The molecular masses of peptides were determined by matrix-assisted laser desorption ionization time-of-fight mass spectrometry on a Voyager-DE STR mass spectrometer (PerSeptive Biosystems). Sequencing of the modified peptide was performed by post-source decay (PSD) measurement on the same instrument. α-Cyano-4-hydroxycinnamic acid was used as the matrix.

N-Terminal Sequencing. Proteins were sequenced by Edman degradation on a Perkin-Elmer Applied Biosystems model 477A Pulsed-Liquid Protein Sequencer. The resulting phenylthiohydantoin (PTH) amino acids were analyzed on-line using an Applied Biosystems 120A PTH analyzer. A PTH-thiaproline standard was prepared by directly loading thiaproline (thiazolidine-4-carboxylic acid) into the sample cartridge of the sequencer.

Stability and Aggregate Analysis of Shh. Circular dichroism (CD) spectra were obtained as described previously (*37*). For gel filtration, 100 μL of 1 mg/mL solutions was injected onto a Superose 12 HR 10/30 FPLC column (Pharmacia) in 20 mM NaH_2PO_4, pH 7.0, 150 mM NaCl (PBS), and 1 mM DTT. The flow rate was 0.5 mL/min, and detection was at 280 nm. Dynamic light scattering measurements were made at 1 mg/mL in the batch mode on a PD2000DLS detector (Precision Detector). Sucrose gradient analysis was carried out by loading 50 μL of 1 mg/mL solutions on 10-40% gradients, with a 0.5 mL 60% sucrose cushion, in PBS, 5 mM DTT. The gradients were centrifuged in an SW60 rotor at 55 000 rpm (300000*g*) for 18 h. The gradients were fractionated by puncturing the bottom of the tubes, and the concentration of Shh proteins in the fractions was determined by ELISA assay. For ELISA, Nunc maxisorp plates were coated with 50 μL/well of anti-Hh monoclonal antibody 5E1 (*39*) at 10 μg/mL in PBS. The plates were incubated overnight at 4 °C, then washed 3 times with PBS, 0.05% Tween 20 (PBST), and blocked with 200 μL/well PBST containing 2% v/v fish skin gelatin (Sigma) for at least 1 h. After blocking, wells were aspirated, and 50 μL of sample, diluted in the blocking buffer, was added. The samples were incubated for 2 h at room temperature, and the plates were aspirated and washed 3 times. A protein A-purified rabbit polyclonal antibody against a 15-mer peptide from the N-terminus of Shh (R. Shapiro, unpublished) diluted in the PBST/gelatin was added at 50 μL/well. After a 90 min incubation at room temperature, wells were washed, and goat anti-rabbit horseradish peroxidase conjugate (BioRad) was added for detection. The conjugate was incubated for 90 min, and the plates were washed, followed by addition of 100 μL of substrate [0.42 mM 3,3′,5,5′-tetramethylbenzidine, 0.005% hydrogen peroxide, and 1% dimethyl sulfoxide in 0.1 M sodium acetate−citrate, pH 4.9] for 2 min before being stopped with 2 N sulfuric acid. The sample absorbances were read on a Molecular Devices SpectraMax Plus plate reader.

Ptc Binding Assay. For each assay, EBNA 293 cells were transiently transfected with truncated, myc-tagged, murine Ptc [(*3*), a gift from Matt Scott, Stanford University] using LIPOFECTAmine (GIBCO/BRL, Grand Island, NY) according to the manufacturer's instructions. Prior to each assay, the concentrations of the test proteins were determined by their absorbance at 280 nm. All binding assay procedures

were carried out at 4 °C. Transfected cells were incubated with serial dilutions of test proteins for 1 h in assay buffer (1% gelatin, 0.2% NaN_3 in PBS, pH 7.2) containing 5 mM DTT. Following one wash with cold assay buffer, 20 nM ShhIg (Shh fused to a human IgG1 Fc domain) was added for 10 min in assay buffer. To avoid potential complications that might arise using a bivalent reporter, we used a form of the ShhIg where one of the Shh arms on the Fc dimer was removed by proteolysis. Cells were then washed twice with cold assay buffer and incubated with phycoerythrin-labeled donkey anti-human Fc (Jackson Immunoresearch) for 30 min. Cells were again washed twice with assay buffer and fixed with 2% *p*-formaldehyde in PBS, and fluorescence was read on a BD FACScan (Becton Dickinson, San Diego, CA).

C3H10T1/2 Cell Assay. The C3H10T1/2 cell line used to quantify the bioactivity of Shh is a murine pluripotent fibroblastic cell line (*40*, *41*) shown previously to differentiate into an osteoblastic phenotype in the presence of Shh (*42*). Shh activity was tested by measuring alkaline phosphatase induction (*43*) in the cultures 5 days after treatment. The assay was performed in a 96-well format, and samples were run in duplicate over a 100-fold range in protein concentration, the appropriate range having been determined in preliminary experiments (*36*). The EC_{50} values were determined graphically and therefore are reported with no more than two significant figures. Unmodified Shh was used as a control in each assay to normalize the EC_{50} values so that interassay comparison among the derivatives was more accurate. The value obtained for unmodified Shh was 99 ± 10 nM in 10 replicate experiments.

RESULTS

Formation of N-Terminal Acyl Amides. Shh was modified with fatty acids at its N-terminus by a reaction mechanism analogous to that utilized by Dawson et al. (*44*) for the ligation of peptide thioesters to peptides with N-terminal cysteines. In the first step, the acyl group of a CoA thioester transfers to the thiol moiety of the N-terminal cysteine through a nucleophilic transesterification reaction. This acyl group then undergoes an intramolecular S to N shift, transferring to the α-amine to form the more stable amide bond (see Figure 1 for structures of the chemically modified forms). The S to N shift regenerates the free thiol on the cysteine so that reaction with a second acyl-CoA is possible. To obtain a singly modified protein, the second thioester adduct was selectively removed by treatment with hydroxylamine. For analytical work, the modified product was then purified by reversed-phase HPLC and characterized for the extent of modification by mass spectrometry. ESI-MS gave a single mass for the purified product corresponding to the addition of a single acyl moiety. To prove that the acyl group was attached to the α-amine, a peptide mapping/sequencing strategy was developed. For these analyses, the free thiol groups on the cysteines were selectively alkylated with 4-vinylpyridine, the protein was digested with endoproteinase Lys-C, and the resulting peptides were separated by reversed-phase HPLC and subjected to mass spectrometry. The 4-vinylpyridine not only provides a mass tag for mass spectrometry but also has a characteristic maximum absorbance at 254 nm that can be monitored by photodiode array detection during the HPLC step. The N-terminal peptide (residues 24−32) was selectively modified by the acyl lipid

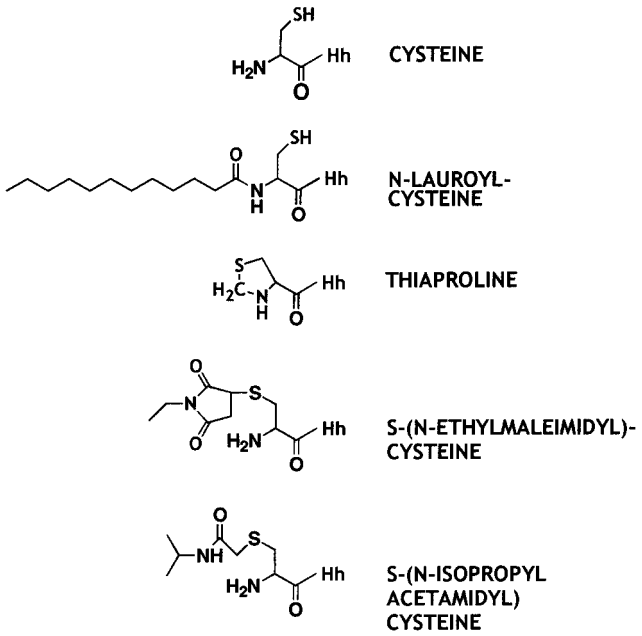

FIGURE 1: Structures of selected chemical modifications of the N-terminal cysteine of Shh.

as evidenced by the disappearance of the corresponding peptide peak and the appearance of a single new peak with a mass corresponding to the N-terminal peptide modified with both the acyl group and 4-vinylpyridine. Thus, it indicates the presence of a free thiol on the N-terminal cysteine prior to modification with 4-vinylpyridine (Figure 2). PSD sequencing of the N-terminal peptide demonstrated that the N-terminal cysteine carried both an acyl and a pyridylethyl group (Figure 3).

Five N-terminally acylated forms of Shh were prepared as described above, containing C_8, C_{10}, C_{12}, C_{14}, or C_{16} acyl groups. The potency of the acylated forms of the Shh proteins was tested by measuring their ability to induce differentiation of C3H10T1/2 cells into osteoblasts. We and others (*23*, *36*, *43*) have observed that C3H10T1/2 cells are Shh-responsive and that Hh activity on this cell line can be readily assessed by measuring the induction of alkaline phosphatase, a marker of mature differentiated osteoblasts. Potency is quantified by determining the concentration of protein required to attain half-maximal induction of alkaline phosphatase. It should also be noted that the more potent forms also induce a higher maximal level of alkaline phosphatase at the end of the 5 day incubation (Figure 4). Histochemical analysis of the C3H10T1/2 cells in the assay (*36*) suggested that the increase in the maximum arises from a larger percentage of the cells responding to the more potent forms (data not shown). The potency of the acylated forms increased as the chain length increased from C_8 to C_{14} (Table 1).

Formation of an N-Terminal Thiazolidine Derivative via Reaction with Formaldehyde. Free cysteine reacts with aldehydes to form thiazolidine ring structures such as thiaproline (Figure 1) in which a methylene bridge links the thiol and the amine [(*45*) and references cited therein]. This reaction chemistry is also applicable to N-terminal cysteines on polypeptides with a free α-amine group. To investigate the effect of this modification on activity, Shh was treated with 0.1% formaldehyde for 1 h at pH 5.5 to generate a reaction product that was separated from unreacted protein

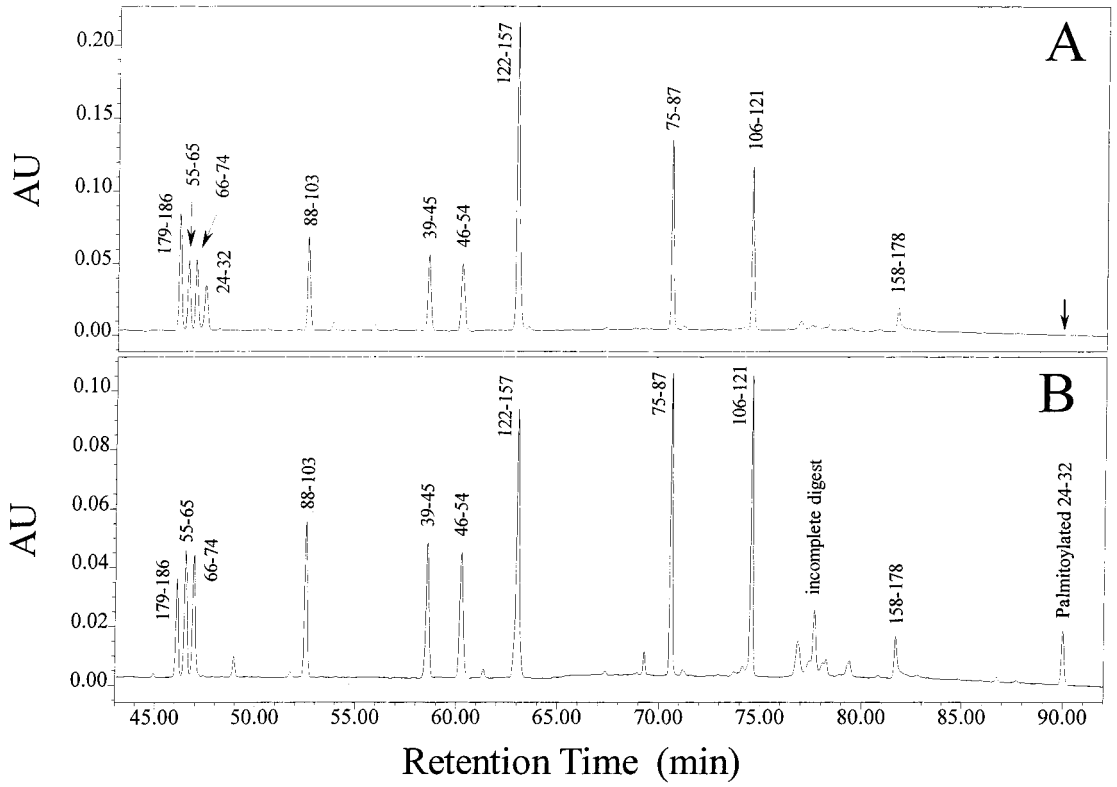

FIGURE 2: Peptide map of unmodified (A) and palmitoylated (B) Shh. The proteins were reduced, alkylated with 4-vinlypyridine, and digested with endoproteinase Lys-C. The resulting peptides were separated by reversed-phase HPLC and identified by MALDI-TOF mass spectrometry. The numbers over each peak indicate the amino acid sequence segment from which the peptide was derived. The unmodified N-terminal peptide segment 24−32 elutes at 47−48 min (A) while for the palmitoylated protein a new peak was found to elute at 90 min (B). Mass spectral analysis showed that the new peak corresponded to the N-terminal peptide, which contained residues 24−32, and was both palmitoylated and alkylated with 4-vinylpyridine.

by cation-exchange chromatography. The molecular mass of the modified product was ~13 Da higher than expected for unmodified Shh, suggesting a single chemical modification. Peptide mapping demonstrated that the site of the modification occurred in the peptide spanning amino acid residues 24−32, and that the exact mass increase was 12 Da. PSD sequencing studies of this peptide (Figure 5) indicated that the modification occurred on the N-terminal cysteine, and could be explained by a modification of the α-amine and the thiol side chain to create the thiaproline form of a thiazolidine. The identification of the thiaproline was confirmed by automated sequencing in which it was shown that the N-terminal residue released by Edman chemistry coeluted on the reversed phase column with a PTH-thiaproline standard (data not shown). In the C3H10T1/2 activity assay, the thiaproline-modified protein was 8-fold more potent than the unmodified Shh (Table 1). While modification of the cysteine in this manner activates the protein, the relative specific activity of the modified protein cannot be determined definitively because the thiazolidine group was found to be unstable at 37 °C (data not shown), and reversal of the reaction to generate unmodified protein may have occurred during incubation in the assay.

Formation of Other Adducts at the N-Terminal Cysteine. Two general thiol modification reagents, maleimides and iodoacetyl compounds, were found to selectively modify the N-terminal cysteine of Shh under mildly acidic conditions, while not reacting with the two internal cysteines. Derivatives tested included *N*-ethylmaleimide, *N*-hydroxyphenylmaleimide, *N*-pyrenylmaleimide, *N*-octylmaleimide, *N*-naphthylmaleimide, iodoacetic acid, and *N*-isopropyliodoacetamide. In each case, the mass spectrum was consistent with a single site of modification. Specific modification of the N-terminal cysteine was confirmed for the *N*-octylmaleimide and *N*-isopropyliodoacetamide modifications by peptide mapping and PSD sequencing (data not shown). When tested in the C3H10T1/2 activity assay, the iodoacetic acid modification abolished activity, the *N*-ethylmaleimide form was unchanged, and the more hydrophobic maleimide modifications were more potent than unmodified Shh (Table 1). The fact that modifications on the α-amine and on the thiol were both capable of activating Shh indicated that the spatial orientation of the modification was not critical.

Substitution of the N-Terminal Cysteine with Hydrophobic Amino Acids. The results of the chemical modifications at the N-terminus of Shh revealed that activity does not require a free thiol on the N-terminal cysteine, despite the fact that this cysteine is conserved among all the Hh proteins. This suggested that mutation of the N-terminal cysteine to a hydrophobic amino acid might also yield a more active protein. In an initial test of this hypothesis, the cysteine was substituted with four amino acids of different hydrophobicities: isoleucine, phenylalanine, tryptophan, or methionine. All four mutants showed increased activity in the C3H10T1/2 assay: the methionine mutant was approximately 2-fold more potent while the isoleucine, tryptophan, and phenylalanine mutants were 3 times more potent than the unmodified protein (Table 2). These results confirmed that the thiol of the N-terminal cysteine is dispensable for activity. In contrast, substitution of the N-terminal cysteine with the hydrophilic

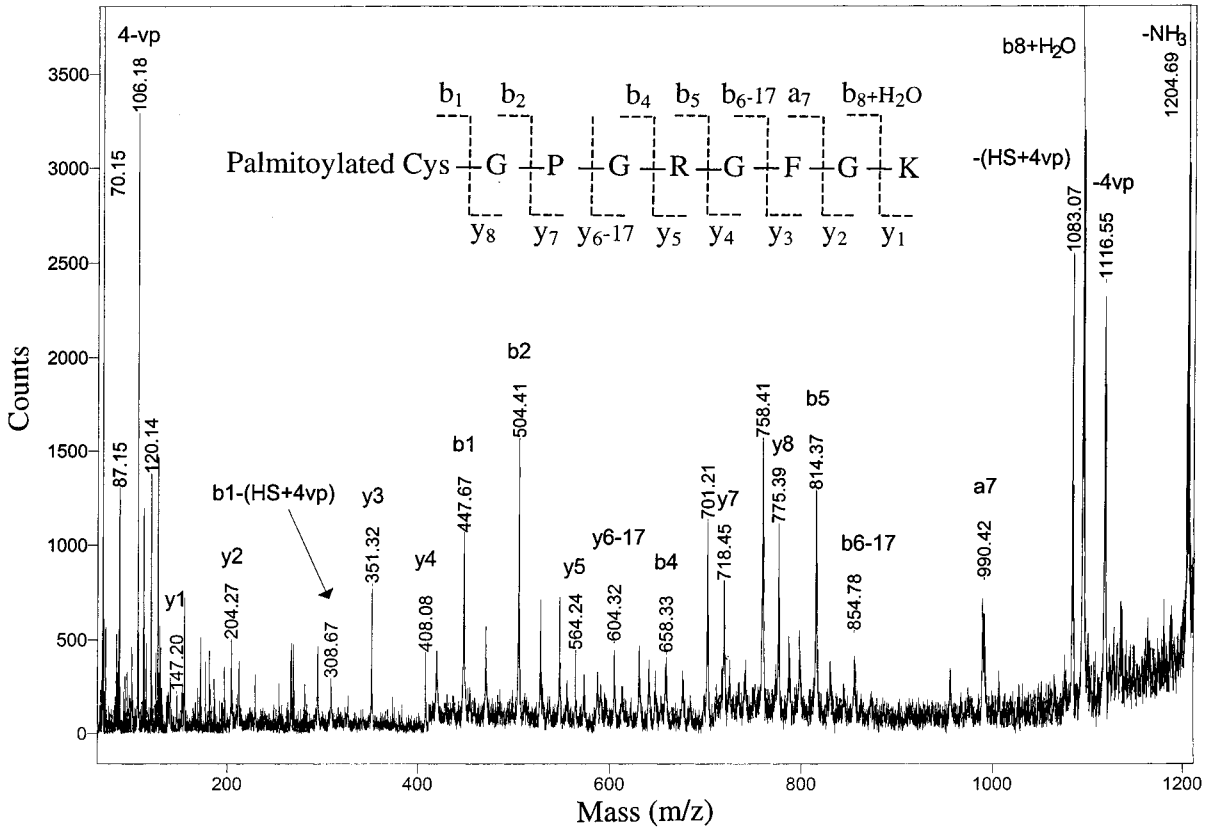

FIGURE 3: PSD mass spectrometry sequencing of the palmitoylated N-terminal peptide of Shh. The peptide eluting at 90 min in Figure 2B was further analyzed by PSD. The y fragments originate from the C-terminus as a result of cleavage between the N−Cα bond, while the a and b fragments originate from the N-terminus as a result of cleavage between the Cα−C and C−N bonds, respectively. The ions are numbered as indicated in the inset scheme. All a and b fragments, including b_1, have a mass corresponding to modification with both palmitate and 4-vinylpyridine. Calculated masses for a_1−a_8 are 419.31, 476.33, 573.38, 630.41, 786.51, 843.53, 990.60, and 1047.62, respectively; for b_1−b_8 are 447.30, 504.33, 601.38, 658.40, 814.50, 871.52, 1018.59, and 1075.61, respectively; and for y_1−y_8 are 147.11, 204.13, 351.20, 408.22, 564.33, 621.35, 718.40, and 775.42, respectively.

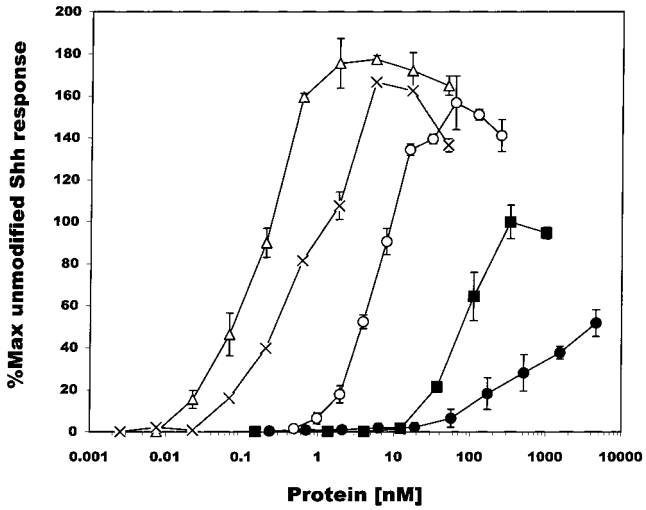

FIGURE 4: Potency of unmodified and N-terminally modified forms of Shh measured by induction of alkaline phosphatase activity in C3H10T1/2 cells. Serial 2-fold dilutions of each protein were incubated with the cells for 5 days and the resulting levels of alkaline phosphatase activity measured at 405 nm using the chromogenic substrate *p*-nitrophenyl phosphate. Symbols: unmodified (■), C24S (●), C24II (○), octylmaleimide-Shh (×), myristoylated-Shh (△).

Table 1: Potency of N-Terminal Chemical Modifications of Shh in the C3H10T1/2 Bioassay

chemical modification	EC_{50} (nM)	relative to unmodified
myristoyl	0.6	160×
palmitoyl	2.5	40×
lauroyl	2.5	40×
N-octylmaleimide	2.5	40×
N-pyrenylmaleimide	5	20×
decanoyl	5	20×
thiaproline	12	8×
N-hydroxyphenylmaleimide	15	7×
N-naphthylmaleimide	15	7×
octanoyl	25	4×
N-isopropylacetamide	50	2×
N-ethylmaleimide	100	1×
unmodified cysteine	99	1×
iodoacetic acid (*S*-carboxymethyl)	>1000	<0.1×

amino acids, aspartic acid, histidine, and serine, almost completely eliminated activity in the C3H10T1/2 assay (Table 2). To further explore the activation of Shh by hydrophobic amino acids, a series of mutants were made with two to six isoleucines at the N-terminus to test whether increasing the number of hydrophobic amino acids would further increase potency. Potency was improved to 8-fold over that of the unmodified protein by replacing the N-terminal cysteine with two isoleucines, but there was no further improvement upon incorporating three or four isoleucines. The mutant proteins containing five and six N-terminal isoleucines precipitated during purification and could not be evaluated for activity. Other di- and tripeptide N-terminal substitutions using combinations of tryptophan,

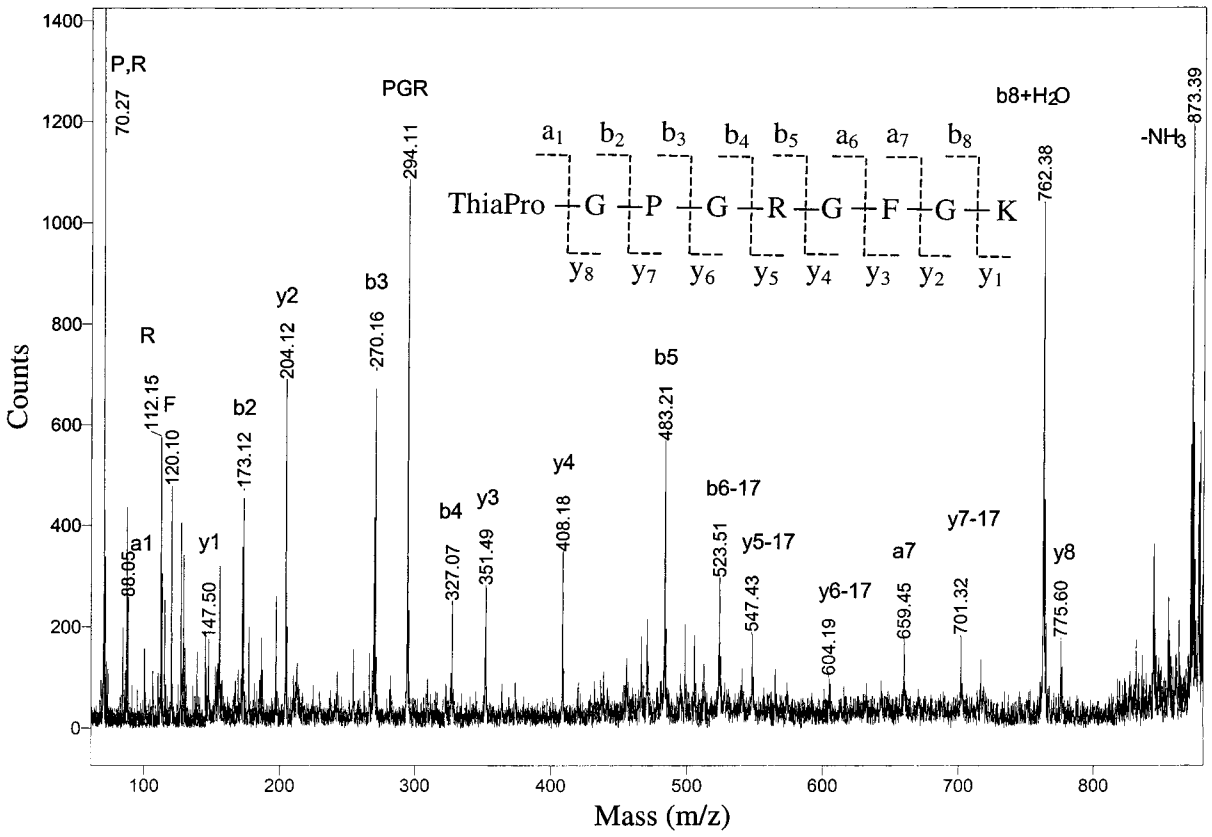

FIGURE 5: PSD mass spectrometry sequencing of the thiaproline-modified N-terminal peptide of Shh. The endoproteinase Lys-C peptide map of the modified protein was generated as described under Experimental Procedures. The thiaproline-modified peptide (residues 24−32) had the same retention time as the peptide from the unmodified protein; however, the mass ion for this peptide was 12 Da greater than expected for an unmodified N-terminal peptide. This peak was further analyzed by PSD. The nomenclature for the ions is given in Figure 3, and the ions are numbered as indicated in the inset scheme. The a and b ions, and most notably the extreme N-terminal a_1 ion, have a mass corresponding to introduction of the thiaproline methylene and were not modified with 4-vinylpyridine. Calculated masses for a_1-a_8 are 88.02, 145.04, 242.10, 299.12, 455.22, 512.24, 659.31, and 716.33, respectively; for b_1-b_8 are 116.02, 173.04, 270.09, 327.11, 483.21, 540.24, 687.30, and 744.33, respectively; and for y_1-y_8 are147.11, 204.13, 351.20, 408.22, 564.33, 621.35, 718.40, and 775.42, respectively.

Table 2: Potency of N-Terminal Hydrophobic Amino Acid Modifications of Shh in the C3H10T1/2 Bioassay

N-terminus	EC_{50} (nM)	relative to unmodified
C24II (2I)	12	8×
C24III (3I)	19	5×
C24IIII (4I)	19	5×
C24IIW	25	4×
C24IW	25	4×
C24F	33	3×
C24I	33	3×
C24FIF	37	3×
C24W	37	3×
C24I, G25I	50	2×
C24M	50	2×
C24 (unmodified)	99	1×
C24H	250	0.4×
C24S	>1000	<0.1×
C24D	>1000	<0.1×

phenylalanine, and isoleucine were also tested, but none were found to be more potent than the di-isoleucine mutation (Table 2). The mutant construct C24I, G25I, in which the first two amino acids of Shh were substituted with di-isoleucine, was expressed to test whether the activities of the poly-isoleucine constructs were constrained by the distance they extended beyond the natural N-terminus. The protein was no more active than C24I, and was substantially less active than the C24II substitution, suggesting that increasing hydrophobicity at the position occupied by the glycine cannot further activate the protein. In addition to hydrophobic modification of Shh, we also introduced a C24II mutation into the Desert Hh N-terminal domain and found the protein was 5−10-fold more potent than unmodified Desert Hh.

Effect of N-Terminal Hydrophobic Modification on the Structure and Stability of Shh. To investigate the effect of hydrophobic modification on the structure of Shh, two modified forms, the C24II mutant and the N-terminal myristoylated protein, were compared to unmodified protein by CD. The CD spectra from 195 to 260 nm for the three proteins were superimposable, demonstrating no major changes in secondary structure (Figure 6A). The stabilities of the proteins were compared by thermal denaturation over the temperature range of 15−70 °C, monitoring by CD at 205 nm. The melting curves for the unmodified protein and C24II mutant were very similar, with 50% denaturation at 56 °C (Figure 6B), while 50% denaturation of the myristoylated form occurred at 52 °C, indicating a small decrease in the stability of the acylated form.

The state of aggregation of the unmodified and some of the modified forms of Shh was compared by gel filtration chromatography. Both the unmodified and C24II proteins eluted as 20 kDa monomeric species. With increasing hydrophobicity from octanoylated, octylmaleimide, and decanoylated forms, the peaks were progressively smaller and

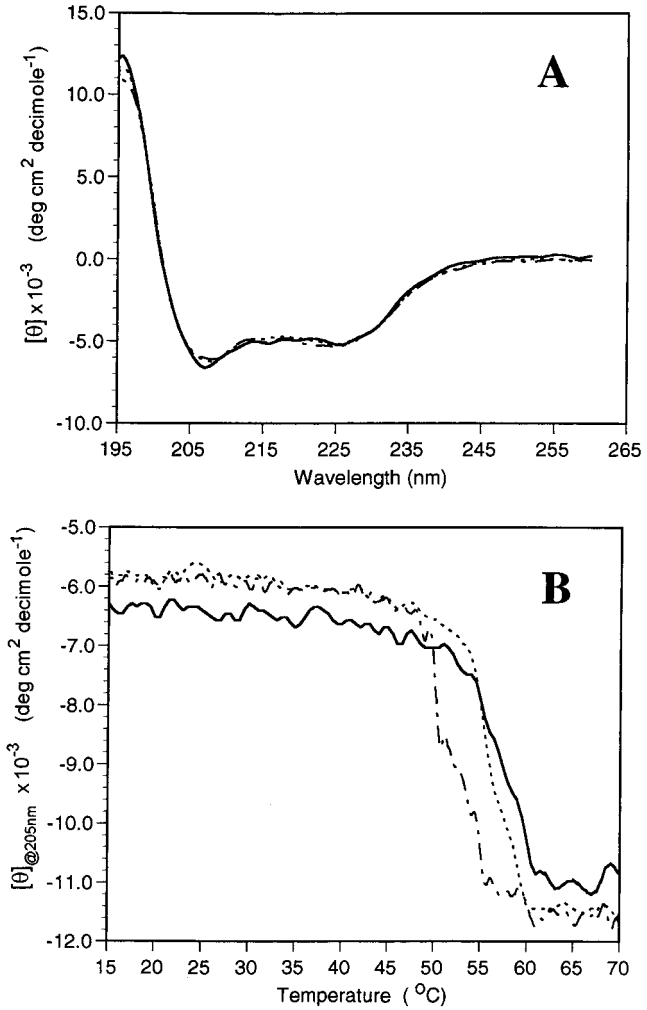

FIGURE 6: CD spectra (A) and thermal denaturation as monitored by CD (B) of the unmodified, myristoylated, and C24II mutant Shh proteins. Procedures are described in Day et al. (*37*). The CD spectra were determined at 20 °C at a scan rate of 10 nm/min. Thermal denaturation was monitored at 205 nm with a linear temperature gradient of 0.5 °C/min. The dashed line is unmodified protein (C24), the solid line is the C24II mutant, and the variable length dashed line is the myristoylated protein.

more delayed and had increasing amounts of a trailing shoulder. The lauroylated, myristoylated, and palmitoylated forms did not elute at all from the column. Thus, there was no indication of aggregated forms of the proteins, but the increasing hydrophobicity appeared to cause irreversible binding to the column. To obtain data on the aggregation state of the myristoylated form, it was compared to the unmodified and C24II proteins by sedimentation velocity centrifugation on 10−40% sucrose gradients. All three proteins were found to sediment in the same fraction. These three proteins and the octylmaleimide form were also analyzed by dynamic light scattering and found to have diameters in the range of 2.9−3.3 nm, consistent with the monomeric state.

Binding of Modified Shh to Cells Expressing the Receptor Ptc. We also tested whether the variations in activity among the N-terminally-modified Shh proteins were accompanied by changes in their affinities for binding to the Hh receptor Ptc. These measurements were made using EBNA 293 cells transiently transfected with a plasmid encoding a form of

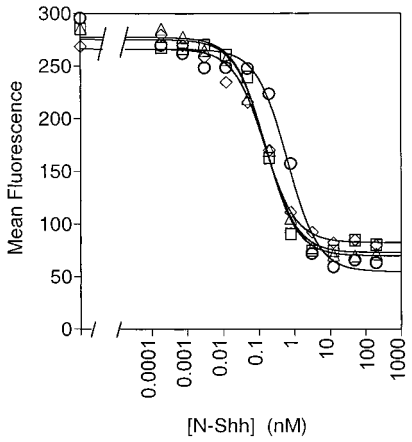

FIGURE 7: Binding of hydrophobically-modified Shh to cells expressing the receptor Ptc. The ability of unmodified (□), myristoylated (○), C24II (△), and C24S (◇) forms of Shh to inhibit the binding of ShhIg fusion protein to EBNA 293 cells transfected with truncated Ptc receptor was measured by FACS analysis as described under Experimental Procedures.

Ptc lacking part of the C-terminal cytoplasmic domain (*3*). Four forms of Shh were tested, unmodified, C24S, C24II, and the myristoylated form, chosen to span a range of potencies in the C3H10T1/2 functional assay. The transfected cells were incubated with each form over a range of concentrations, and residual free binding sites were measured by exposing the cells to a ShhIg fusion protein, the binding of which was quantified by flow cytometry using a phycoerytherin-labeled anti-Fc detection reagent. Control experiments showed that there was no measurable re-equilibration of Shh binding during the brief incubation with the ShhIg probe. Figure 7 shows that all four forms of Shh, which differ in potency in the C3H10T1/2 assay by greater than 1600-fold, show similar affinities in this binding assay. In three replicate experiments, the maximum range found was 10-fold among the four forms. In interpreting the data from Figure 7, it is important to note that these are relatively high-affinity ligands binding to a highly overexpressing cell line. Control experiments suggested that the total concentration of surface receptor present in the assay is significant in comparison to the IC_{50} values being measured (data not shown). This situation raises the possibility that the observed variation in IC_{50} values might not fully reflect the true variation in the underlying binding affinities, because even a very high affinity ligand must still be present at half the total receptor concentration in order to give 50% inhibition of ShhIg binding. With this possible limitation, the results from Figure 7 show that the large variations in potency among the four forms of Shh represented are not accompanied by any detectable variations in the affinity with which these proteins bind to the receptor Ptc. Furthermore, the low nanomolar affinity measured in this assay is lower than the EC_{50} measured in the C3H10T1/2 cell activity assay for all the proteins except the myristoylated form. Additional comparisons between the binding of Shh and the myristoylated form were performed in which the buffer conditions were varied by replacing gelatin protein with either bovine serum albumin or milk protein, or by omitting DTT. In all cases, these two forms of Shh, which possess widely different functional activities, gave virtually indistinguishable IC_{50} values in the binding assay.

Hydrophobic Modifications at Other Positions on Shh. During the processing of the Shh precursor in cells, the protein becomes modified with a cholesterol moiety at or near the C-terminus (*22*), and most of the protein is also modified with a palmitic acid at the N-terminus (*23*). The protein with both a C-terminal cholesterol and an N-terminal palmitic acid, as well as that with an N-terminal palmitic acid alone, was found to be more active than the unmodified form of the protein (*23*), but the contribution of the C-terminal cholesterol to this activation is unclear. In order to try to determine if hydrophobic modifications at the C-terminus would produce an enhancement of activity, a cysteine was added near the C-terminus for subsequent modification with hydrophobic maleimide derivatives. Two constructs were made, one with a cysteine at position 192 (A192C) and one at position 197, the mature C-terminus (G197C). The constructs also had the C24II modification so that the N-terminal cysteine would not be available for maleimide modification. These C-terminal mutations did not alter the bioactivity of the protein compared to the wild-type sequence. Modification of the C-terminal cysteines with *N*-(1-pyrenyl)maleimide and *N*-(octyl)maleimide was found to activate the protein by 2−3-fold over the C24II parent construct, much smaller than the 20-fold effect of the same modifications of C24 (Table 1). To rule out the possibility that the C24II mutation in this construct might have interfered with the activating properties of the C-terminal modification, another construct was made where C24 was mutagenized to an alanine instead of the di-isoleucine. Again, pyrene modification at the C-terminus had only a modest 2-fold effect on activity. The aggregation state of the C192 and C197 octylmaleimide-modified forms was analyzed by dynamic light scattering at a protein concentration of 1 mg/mL. Initially, the proteins had the same diameter of ∼3 nm found for the N-terminally modified forms, but with continued analysis the diameter rose as high as 15 nm. We suspect that a rise in temperature in the cuvette may have induced this aggregation, and we found that cooling the samples back to 4 °C reversed the effect. It is not clear if this effect is relevant to the state of the proteins in the bioassay where the EC_{50} is only 4 nM (80 ng/mL).

DISCUSSION

Shh undergoes two unusual post-translational processing events that result in the covalent addition of cholesterol at the C-terminus (*22*) and a long-chain fatty acid on the N-terminal cysteine (*23*). In addition to tethering the protein to the surface of the cell in which it is synthesized, lipid modification greatly activates the protein in Hh-responsive cell-based signaling assays. We report here a detailed investigation of the types and locations of hydrophobic adducts that increase Shh signaling activity in C3H10T1/2 cells.

Examples of some of the chemical structures appended to the N-terminus of Shh are depicted in Figure 1. The relative potencies of the chemically-modified forms and the amino acid substituted forms in the C3H10T1/2 signaling assay are compared in Tables 1 and 2, respectively. The results show that a wide variety of hydrophobic modifications can lead to activation of the protein. These modifications include long-chain fatty acids (C_8−C_{16}), heterochemical ring structures (thiazolidines), aromatics (phenylalanine, pyrene), branched hydrocarbons (isoleucine), and a heterochemical aliphatic group (methionine). Conversely, hydrophilic modifications such as the addition of acidic, basic, or hydroxyl functions substantially decrease Shh activity. These results suggest that modification of Shh by any method that increases the hydrophobic nature of the N-terminus may enhance the biological activity of the protein. The data also suggest that the exact position of the hydrophobic modification on the N-terminal amino acid is not critical for activation. Modification can be on either the α-amine or the thiol of the N-terminal cysteine, as exemplified by fatty acid amidation of the α-amine and *N*-alkylmaleimide derivatization of the thiol.

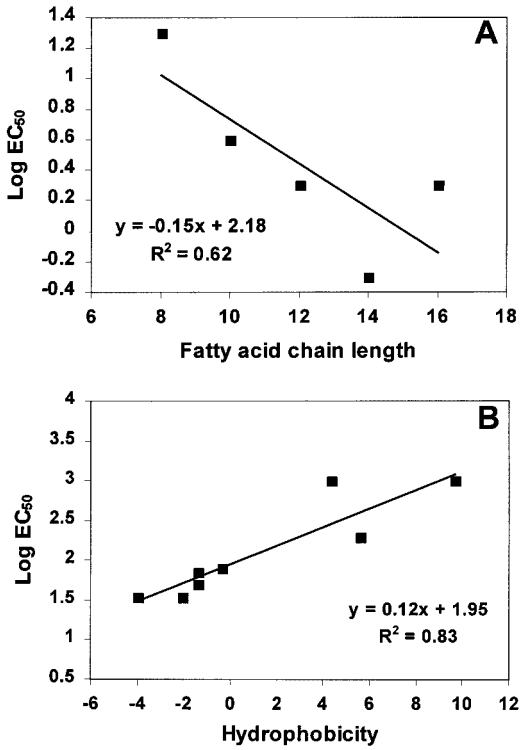

FIGURE 8: Correlation of the potency of the modified forms of Shh in the C3H10T1/2 cell-based assay to the chain length of the fatty acid adduct (A) or the hydrophobicity of the amino acid substitution at C24 (B). EC_{50} values are from Tables 1 and 2. The hydrophobicity values for the amino acids are from references (*46*, *47*).

The degree of activation appears to generally correlate with the relative hydrophobicity of the modification. One example of this trend is given by the fatty acid modifications, wherein activity increases with chain length in the C_8−C_{14} series (Figure 8A) with a correlation coefficient of 0.97. In our analysis, the C_{16} form had less activity than the C_{14} form, perhaps because of solubility or stability problems in the assay, reducing the overall correlation coefficient as shown in the figure. Another example is the amino acid substitutions in which the order of activity (Phe = Ile = Trp > Met > Cys > His > Ser = Asp) is comparable to that of amino acid hydrophobicity scales (*46*, *47*) (Figure 8B). The relative hydrophobicity of the other chemical adducts has not been measured, and it is possible that other features of the modifying group, aside from hydrophobicity, can have some effect on activation.

In contrast to the tolerance for structural variability at the N-terminal cysteine, there is a spatial component to activation apparent in the chain-length dependence of multiple hydro-

phobic amino acids near the N-terminus. It was found that two hydrophobic amino acids were more potent than one, but addition of three or four hydrophobic amino acids did not further increase potency although the protein had not yet reached the maximum activation found with the long-chain fatty acids. In addition, increasing the hydrophobicity of the second amino acid in the sequence from glycine to isoleucine did not increase the activity of the construct. This result is in keeping with other structure−function work that highlights the importance of the length and sequence of the first 15 amino acids of Shh, which are highly conserved among all Hh proteins (E. Garber, manuscript in preparation).

The site of hydrophobic modification on the protein as a whole also appears to be important. Modification of the N-terminal cysteine with *N*-(1-pyrenyl)maleimide or *N*-(1-octyl)maleimide results in more potent proteins than similar modifications at cysteines engineered at positions 192 and 197 near the C-terminus. We have also tested whether a hydrophobic moiety activates Shh if it is attached at nine other positions surrounding the surface of the protein where a cysteine had been substituted for the natural amino acid [see Pepinsky et al. (*38*) for modification sites]. No activation was found after modification of each site with *N*-(1-pyrenyl)-maleimide. If there is a preference for activation at the N-terminal cysteine the reason may be the unusual structure of this region. The crystal structure of the N-terminal signaling domain shows the N-terminal 15 amino acids extending outward to make contact with a hydrophobic pocket on a neighboring Shh molecule in the crystal (*38*). Whether this interaction has any physiological significance is unknown, but it suggests that the hydrophobic additions at the N-terminus may act at a site up to 50 Å from the body of the protein, in contrast to the more proximal location of the other modification sites tested. It is also intriguing that the C-terminal modifications are partially activating since the N- and C-termini of the protein extend from the same face of the Shh structure. While these data highlight the effectiveness of N-terminal modification for activation, the relative importance of the natural lipid modifications at the N- and C-termini is not clear. We were unable to produce the form of the protein with the natural cholesterol modification at the C-terminus and an unmodified N-terminus because most of the unmodified N-terminus is proteolytically cleaved at the N-9 and N-10 basic amino acid residues when expressed in insect and mammalian cells. Thus, we do not know if the natural cholesterol modification contributes in a more significant fashion to activation.

What insight can be gained about the mechanism of activation from these results? When Shh, modified with both a long-chain fatty acid and a cholesterol, was first isolated as a detergent extract from cells (*23*), it seemed possible that activation was due to the aggregation of the isolated protein, increasing the avidity of binding to its receptor, Ptc. However, the observation that a single hydrophobic group can activate Shh makes this mechanism unlikely. Experiments by Pool and Thompson (*48*) with bovine pancreatic trypsin inhibitor modified with a single N-terminal fatty acid have shown that the concentration required to form aggregates of the palmitoylated protein was above 11 μM, while a myristoylated protein did not form aggregates even at 5 mM. These concentrations are well above that required for signaling in the C3H10T1/2 cell assay ($EC_{50} = 0.5-40$ nM). Modification by addition of one or two isoleucines at the N-terminus would seem even less likely to cause aggregation. It is possible that Shh has some propensity to aggregate that is aided by hydrophobic modification, but gel filtration, light scattering, and sucrose gradient sedimentation data for the C24II and myristoylated forms showed no evidence for higher molecular weight species. Since in some instances hydrophobic modification of proteins can result in changes in protein structure or catalytic activity (*49*, *50*), we tested the effect of hydrophobic modification on Shh structure and thermal stability by CD measurements. No change in secondary structure and only a small decrease in thermal stability were found for the myristoylated form compared to the unmodified protein (Figure 6).

Another possible explanation for the increased potency of the hydrophobic forms of Shh would be a higher affinity for its receptor. The best characterized receptor for Hh is the multi-pass membrane-spanning protein Ptc, which modulates signaling by the seven-transmembrane protein Smoothened in a manner that is regulated by the binding of Hh to Ptc (*3*−*6*, *20*). We were unable to demonstrate any substantial differences in the receptor binding affinities of different modified forms of Shh in an assay using cells transfected with the Ptc protein. All the Shh forms tested showed a nearly equal high affinity for the Ptc protein expressed on the surface of EBNA 293 cells, even though their potency in the C3H10T1/2 assay varied over a 1600-fold range. Only the most potent hydrophobic form, that modified with a long-chain myristoyl fatty acid, had an EC_{50} value in the signaling assay that appears commensurate with its high affinity for Ptc.

The discordance between binding affinity to the receptor Ptc and signaling potency is puzzling. One trivial explanation might be that Shh is signaling through another receptor besides Ptc in C3H10T1/2 cells. We cannot rule out this possibility at present because we do not have a way to block binding to specific receptors. However, our previous results have shown that signaling by Shh upregulates the expression of the normal components of the Hh pathway, Gli1 and Ptc, in C3H10T1/2 cells (*36*), suggesting that Ptc is the primary signaling receptor. Another possible explanation for the discrepancy between the two assays could involve differences in presentation of the receptor. The Ptc binding assay is conducted with cells overexpressing a C-terminally truncated form of Ptc. The high levels of Ptc in this assay, the structural effect of the C-terminal truncation (*51*), or the relatively low amount of Smoothened compared to Ptc might alter the Shh binding properties of Ptc on the transfected cells compared to that present in the C3H10T1/2 cells. Stone et al. (*3*) reported that cotransfection of cells with Ptc and Smoothened did not alter the apparent binding affinity of Shh but their immunoprecipitation data suggested that only 30% of the Ptc was associated with Smoothened, and such an effect might have been missed. Thus, the lack of correlation suggests that a critical aspect of the binding or signaling mechanism is absent in the Ptc binding assay. We have attempted to perform receptor binding assays using the C3H10T1/2 cells but have been unable to detect specific binding to the small amount of Ptc above the level of nonspecific binding, presumably because of the relatively low level of receptor expression on these cells.

An important clue to the mechanism by which N-terminal hydrophobic modifications increase the activity of Shh may lie in the observation that, in addition to their higher potency as measured by EC_{50}, the hydrophobically-modified forms also induce a higher maximal level of alkaline phosphatase in the signaling assay than the less active forms (Figure 4). Moreover, the hydrophilic, least active, forms of hedgehog can inhibit the activity of the hydrophobic forms when added at high concentrations (data not shown). This is similar to the antagonistic activity of N-terminally-truncated forms of Shh proteins found by us (*36*) and others (*52*). These results suggest that in this assay the less active forms act as partial antagonists; that is, they are able to bind the same receptor as the hydrophobically-modified forms, but once bound are less efficient at activating the receptor to induce subsequent intracellular signals.

Even though the hydrophobic modifications aid in transducing the Shh signal to Ptc, our structure−activity results do not support a mechanism involving specific binding interactions of the hydrophobic moeity. Such specific interactions would be expected to be dependent on the presence of particular structural features in the interacting molecules. Among the activating moieties identified here, however, no specific features seem discernible other than the general correspondence to hydrophobicity. This is most striking in comparing the proteins modified with an octanoyl, thiaproline, *N*-isopropylacetamide, or C24II substitution, which have similar potencies despite their large differences in chemical structure. These results suggest that the binding interactions may be with a general hydrophobic area, perhaps even membrane lipids, at a specific distance from the body of the Shh protein as suggested by the results of the isoleucine substitutions described above.

Activation of Shh by hydrophobic modification has been shown to occur in three different cell lines: pluripotent C3H10T1/2 fibroblast cells, TM3 mouse Leydig cells (K. Williams, unpublished observations), and the bone marrow stromal line M210B4 (D. Olson, personal communication). We are currently investigating whether hydrophobic activation is unique to these kinds of in vitro assay systems, or whether it is a more general phenomenon that is relevant to the effects of Shh in vivo. The myristoylated protein is also significantly more potent than the unmodified protein at inducing ventral forebrain neurons in explants of embryonic stage E11 rat brain telencephalon (J. Kohtz and G. Fishell, personal communication). In vivo, activation has been tested in a system where treatment with Shh protects adult rat striatum from the excitotoxic effects of malonate injection. In this model, increased potencies found for both the myristoylated form (100-fold) (*53*) and the C24II mutant (10-fold) (T. Engber, personal communication), compared to unmodified Shh, directly correlate with increases in potency in the C3H10T1/2 assay. In contrast, no difference in potency between lipid-modified and unmodified Shh was observed in an assay measuring increases in islet-1 expression in chick embryo neural plate explants (*23*). In this assay, even truncated forms that act as antagonists in the C3H10T1/2 assay are equal in potency to the unmodified protein (*36*). The difference in the apparent mechanism of this assay is not understood.

Selective chemical modification of Shh was aided by the unusual reactivity of the N-terminal cysteine. The proximity of the free α-amine to the thiol group on the side chain allowed us to exploit two unusual reaction mechanisms. One of these is the reaction with thioester groups, such as the acyl-CoA derivatives, in which the exchange of the acyl ester onto the cysteine thiol is followed by a rapid S to N shift of the acyl group to form the stable amide at the α-amine (*44*). We found that acylation by this mechanism worked well with long-chain fatty acyl-CoA esters but was much less efficient with acyl chain lengths less than 12 carbons. The reason for this observation may be that the reaction is accelerated by the binding of the positively charged Shh protein (calculated pI = 8.8) to the negatively charged CoA micelles, and thus the reaction rate is dependent on the critical micelle concentration of the CoA derivatives. The critical micelle concentration for lauroyl-CoA (C_{12}) has been reported to be ~1 mM (*54*), close to the concentration of acyl-CoA derivatives in the reactions reported herein. With this possibility in mind, acylation with decanoyl- and octanoyl-CoA was greatly improved by increasing their concentrations in the reactions. Further evidence for the acceleration of the reaction by micelle formation, in addition to the chain-length dependence, is provided by the observation that the reaction is inhibited by 1% octyl-β-D-glucopyranoside, which would be expected to disrupt micelle formation. The reaction rate was also reduced by increasing the salt concentration, which would be expected to inhibit the ionic interaction of Shh with the micelles (D. Baker and F. Taylor, unpublished observations).

The second unusual chemical mechanism utilized here is the reaction of the N-terminal cysteine with aldehydes to form thiazolidine adducts linking the α-amine and thiol groups in a ring structure (Figure 1) (*45*). Thiazolidine modification of the N-terminal cysteine was discovered on Shh expressed in *Pichia pastoris*, presumably from reaction with formaldehyde generated by the metabolism of the methanol carbon source (data not shown). An unexpected observation was that the thiaproline adduct formed by reaction with formaldehyde was not stable; the half-life of the thiaproline adduct was less than 24 h when incubated at 37 °C in sodium phosphate buffer at pH 5.5. This is contrary to the suggestion that this ring structure should be stable (*55*), and indicates that this chemistry might not be useful for the long-term modification of proteins.

In addition to these unusual reaction chemistries, the N-terminal cysteine of Shh was also preferentially reactive with other standard thiol modification reagents, such as maleimides and alkyl halides, compared to the two other cysteines in the Shh sequence. This is presumably due to the greater exposure of the cysteine at the N-terminus compared to the more buried locations of the other cysteines (*56*, A. Boriack-Sjodin, unpublished results).

An N-terminal cysteine is conserved in all known Hh protein sequences including fish, frog, insect, bird, and mammals. Our finding that this cysteine in both Shh and Desert Hh can be replaced with other hydrophobic amino acids suggests that the cysteine does not have a direct role in signaling activity, but instead has the sole purpose of serving as a site for modification with the fatty acid adducts required for membrane tethering and activation. In addition to the possible utility of these modifications in elucidation of the mechanism by which Shh exerts its biological function, there are practical implications for the manufacture and

therapeutic use of Shh. The susceptibility of the N-terminal cysteine to oxidation or other chemical modifications is an impediment to the expression, purification, and formulation of recombinant forms of the protein. Replacement with hydrophobic amino acids or protection by chemical modification solves these production problems, while at the same time creating more potent molecules. The choice of modification also potentially provides a means of optimizing the activity, solubility, pharmacokinetics, and pharmacodynamics of the protein.

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REFERENCES

1. Hammerschmidt, M., Brook, A., and McMahon, A. P. (1997) *Trends Genet. 13*, 14−21.
2. Ingham, P. W., Taylor, A. M., and Nakano, Y. (*1*991) *Nature 353*, 184−187.
3. Stone, D. M., Hynes, M., Armanini, M., Swanson, T. A., Gu, Q., Johnson, R. L., Scott, M. P., Pennica, D., Goddard, A., Phillips, H., Noll, M., Hooper, J. E., de Sauvage, F., and Rosenthal, A. (1996) *Nature 384*, 129−134.
4. Marigo, V., Davey, R. A., Zuo, Y., Cunningham, J. M., and Tabin, C. J. (1996) *Nature 384*, 176−179.
5. Chen, Y., and Struhl, G. (1996) *Cell 87*, 553−563.
6. Ingham, P. W. (1998) *EMBO J. 17*, 3505−3511.
7. Motoyama, J., Takabatake, T., Takeshima, K., and Hui, C. (1998) *Nat. Genet. 18*, 104−106.
8. Carpenter, D., Stone, D. M., Brush, J., Ryan, A., Armanini, M., Frantz, G., Rosenthal, A., and de Sauvage, F. J. (1998) *Proc. Natl. Acad. Sci. U.S.A. 95*, 13630−13634.
9. Zaphiropoulos, P. G., Unden, A. B., Rahnama, F., Hollingsworth, R. E., and Toftgard, R. (1999) *Cancer Res. 59*, 787−792.
10. Chuang, P. T., and McMahon, A. P. (1999) *Nature 397*, 617−621.
11. Hooper, J. E., and Scott, M. P. (1989) *Cell 59*, 751−765.
12. Nakano, Y., Guerrero, I., Hidalgo, A., Taylor, A., Whittle, J. R., and Ingham, P. W. (1989) *Nature 341*, 508−513.
13. Carstea, E. D., Morris, J. A., Coleman, K. G., Loftus, S. K., Zhang, D., Cummings, C., Gu, J., Rosenfeld, M. A., Pavan, W. J., Krizman, D. B., Nagle, J., Polymeropoulos, M. H., Sturley, S. L., Ioannou, Y. A., Higgins, M. E., Comly, M., Cooney, A., Brown, A., Kaneski, C. R., Blanchette-Mackie, E. J., Dwyer, N. K., Neufeld, E. B., Chang, T. Y., Liscum, L., Tagle, D. A., et al. (1997) *Science 277*, 228−231.
14. Loftus, S. K., Morris, J. A., Carstea, E. D., Gu, J. Z., Cummings, C., Brown, A., Ellison, J., Ohno, K., Rosenfeld, M. A., Tagle, D. A., Pentchev, P. G., and Pavan, W. J. (1997) *Science 277*, 232−235.
15. Incardona, J. P., and Eaton, S. (2000) *Curr. Opin. Cell Biol. 12*, 193−203.
16. Incardona, J. P., Gaffield, W., Kapur, R. P., and Roelink, H. (1998) *Development 125*, 3553−3562.
17. Cooper, M. K., Porter, J. A., Young, K. E., and Beachy, P. A. (1998) *Science 280*, 1603−1607.
18. Taylor, A. M., Nakano, Y., Mohler, J., and Ingham, P. W. (1993) *Mech. Dev. 42*, 89−96.
19. Tabata, T., and Kornberg, T. B. (1994) *Cell 76*, 89−102.
20. Denef, N., Neubuser, D., Perez, L., and Cohen, S. M. (2000) *Cell 102*, 521−531.
21. Beachy, P. A., Cooper, M. K., Young, K. E., von Kessler, D. P., Park, W. J., Hall, T. M., Leahy, D. J., and Porter, J. A. (1997) *Cold Spring Harbor Symp. Quant. Biol. 62*, 191−204.
22. Porter, J. A., Young, K. E., and Beachy, P. A. (1996) *Science 274*, 255−259.
23. Pepinsky, R. B., Zeng, C., Wen, D., Rayhorn, P., Baker, D. P., Williams, K. P., Bixler, S. A., Ambrose, C. M., Garber, E. A., Miatkowski, K., Taylor, F. R., Wang, E. A., and Galdes, A. (1998) *J. Biol. Chem. 273*, 14037−14045.
24. Bumcrot, D. A., Takada, R., and McMahon, A. P. (1995) *Mol. Cell. Biol. 15*, 2294−2303.
25. Lee, J. J., Ekker, S. C., von Kessler, D. P., Porter, J. A., Sun, B. I., and Beachy, P. A. (1994) *Science 266*, 1528−1537.
26. Porter, J. A., von Kessler, D. P., Ekker, S. C., Young, K. E., Lee, J. J., Moses, K., and Beachy, P. A. (1995) *Nature 374*, 363−366.
27. Porter, J. A., Ekker, S. C., Park, W. J., von Kessler, D. P., Young, K. E., Chen, C. H., Ma, Y., Woods, A. S., Cotter, R. J., Koonin, E. V., and Beachy, P. A. (1996) *Cell 86*, 21−34.
28. Burke, R., Nellen, D., Bellotto, M., Hafen, E., Senti, K. A., Dickson, B. J., and Basler, K. (1999) *Cell 99*, 803−815.
29. Rietveld, A., Neutz, S., Simons, K., and Eaton, S. (1999) *J. Biol. Chem. 274*, 12049−12054.
30. Roelink, H., Porter, J. A., Chiang, C., Tanabe, Y., Chang, D. T., Beachy, P. A., and Jessell, T. M. (1995) *Cell 81*, 445−455.
31. Fan, C. M., Porter, J. A., Chiang, C., Chang, D. T., Beachy, P. A., and Tessier-Lavigne, M. (1995) *Cell 81*, 457−465.
32. Strigini, M., and Cohen, S. M. (1997) *Development 124*, 4697−4705.
33. Bellaiche, Y., The, I., and Perrimon, N. (1998) *Nature 394*, 85−88.
34. Ramirez-Weber, F. A., and Kornberg, T. B. (1999) *Cell 97*, 599−607.
35. Christian, J. L. (2000) *Curr. Opin. Cell Biol. 12*, 244−249.
36. Williams, K. P., Rayhorn, P., Chi-Rosso, G., Garber, E. A., Strauch, K. L., Horan, G. S., Reilly, J. O., Baker, D. P., Taylor, F. R., Koteliansky, V., and Pepinsky, R. B. (1999) *J. Cell Sci. 112*, 4405−4414.
37. Day, E. S., Wen, D., Garber, E. A., Hong, J., Avedissian, L. S., Rayhorn, P., Shen, W., Zeng, C., Bailey, V. R., Reilly, J. O., Roden, J. A., Moore, C. B., Williams, K. P., Galdes, A., Whitty, A., and Baker, D. P. (1999) *Biochemistry 38*, 14868−14880.
38. Pepinsky, R. B., Rayhorn, P., Day, E. S., Dergay, A., Williams, K. P., Galdes, A., Taylor, F. R., Boriack-Sjodin, P. A., and Garber, E. A. (2000) *J. Biol. Chem. 275*, 10995−11001.
39. Ericson, J., Morton, S., Kawakami, A., Roelink, H., and Jessell, T. M. (1996) *Cell 87*, 661−673.
40. Reznikoff, C. A., Brankow, D. W., and Heidelberger, C. (1973) *Cancer Res. 33*, 3231−3238.
41. Taylor, S. M., and Jones, P. A. (1979) *Cell 17*, 771−779.
42. Nakamura, T., Aikawa, T., Iwamoto-Enomoto, M., Iwamoto, M., Higuchi, Y., Pacifici, M., Kinto, N., Yamaguchi, A., Noji, S., Kurisu, K., Matsuya, T., and Maurizio, P. (1997) *Biochem. Biophys. Res. Commun. 237*, 465−469.
43. Kinto, N., Iwamoto, M., Enomoto-Iwamoto, M., Noji, S., Ohuchi, H., Yoshioka, H., Kataoka, H., Wada, Y., Yuhao, G., Takahashi, H. E., Yoshiki, S., and Yamaguchi, A. (1997) *FEBS Lett. 404*, 319−323.
44. Dawson, P. E., Muir, T. W., Clark-Lewis, I., and Kent, S. B. (1994) *Science 266*, 776−779.
45. Kallen, R. G. (1971) *J. Am. Chem. Soc. 93*, 6236−6248.
46. Radzicka, A., and Wolfenden, R. (1988) *Biochemistry 27*, 1664−1670.
47. Creighton, T. E. (1993) *Proteins. Structures and Molecular Properties*, 2nd ed., W. H. Freeman and Company, New York.
48. Pool, C. T., and Thompson, T. E. (1998) *Biochemistry 37*, 10246−10255.
49. Stoffel, R. H., Inglese, J., Macrae, A. D., Lefkowitz, R. J., and Premont, R. T. (1998) *Biochemistry 37*, 16053−16059.
50. Resh, M. D. (1996) *Cell. Signalling 8*, 403−412.

51. Johnson, R. L., Milenkovic, L., and Scott, M. P. (2000) *Mol. Cells 6*, 467−478.
52. Katsuura, M., Hosono-Sakuma, Y., Wagatsuma, M., Yanagisawa, S., Okazaki, M., and Kimura, M. (1999) *FEBS Lett. 447*, 325−328.
53. Engber, T. M., Wang, S., Huang, C., Reilly, J., Galdes, A., and Mahanthappa, N. (1999) *Abstract, Society for Neuroscience 1999 Annual Meeting*.
54. Smith, R. H., and Powell, G. L. (1986) *Arch. Biochem. Biophys. 244*, 357−360.
55. Zhang, L., and Tam, J. P. (1996) *Anal. Biochem. 233*, 87−93.
56. Hall, T. M., Porter, J. A., Beachy, P. A., and Leahy, D. J. (1995) *Nature 378*, 212−216.

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