A Highly Conserved Amino-terminal Region of Sonic Hedgehog Is Required for the Formation of Its Freely Diffusible Multimeric Form*

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Although members of the Hedgehog (Hh) family were initially described as morphogens, many of these early conclusions were based on experiments that used non-physiologically relevant forms of Hh. Native Hh is modified by cholesterol (HhNp) and palmitate. These hydrophobic modifications are responsible for the ability of Hh to associate with cellular membranes, a property that initially appeared inconsistent with its ability to act far from its site of synthesis. Although it is now clear that Hh family members are capable of acting directly in long-range signaling, the form of Hh capable of this activity remains controversial. We have previously provided evidence for a freely diffusible multimeric form of Sonic Hedgehog (Shh) termed s-ShhNp, which is capable of accumulating in a gradient fashion through a morphogenetic field. Here, we provide further evidence that s-ShhNp is the physiologically relevant form of Shh. We show that the biological activity of freely diffusible ShhNp resides in its multimeric form and that this multimeric form is exceedingly stable, even to high concentrations of salt and detergent. Furthermore, we now validate the Shh-Shh interactions previously observed in the crystal structure of human Shh, showing that a highly conserved amino-terminal domain of Shh is important for the formation of s-ShhNp. We also conclusively show that palmitoylation is required for s-ShhNp formation. Thus, our results identify both protein-protein and protein-lipid interactions that are required for s-ShhNp formation, and provide the first structural analyses supporting the existence of Shh multimers.

The Hedgehog (Hh)3 family of proteins plays an important instructive role in metazoan development (1). More recently, the human Hh family members Sonic Hedgehog (Shh) and Indian Hedgehog were shown to play an important role as mitogens and survival factors for the growth of numerous types of tumor cells (2–4). Although the physiological responses and signaling pathway of Hh are well studied, less is known about how Hh family members function as biological ligands. Questions as to the state of the physiologically relevant ligand, how the ligand moves through extracellular space, and even how it exits cells to reach the plasma membrane, remain unanswered (5, 6). One reason for these remaining questions is the unusual biochemistry of the Hh family of proteins. Hh is synthesized as a protein of ~45 kDa, which subsequently undergoes an intramolecular proteolytic cleavage into an amino-terminal domain (HhN) and a carboxyl-terminal domain (HhC) (7, 8). During this cleavage, the amino-terminal domain is post-translationally modified by the covalent addition of cholesterol (HhNp), with the carboxyl-terminal domain acting as a cholesterol transferase (9). HhNp is sufficient to activate all known Hh-dependent processes (10–15). HhNp is also modified by the covalent addition of a second lipid, palmitate, to its extreme amino terminus (16). This second lipid modification is added to Hh by a protein termed Skinny Hedgehog (Skn) (17–20), which has homology to a family of O-acyl transferases. The exact functions of these lipid modifications to HhN remain uncertain. However, it has been suggested that cholesterol plays a role in localizing Hh to specific micro-domains on the plasma membrane (21), whereas the palmitate on Hh may play a role in increasing its potency (22) or movement (23).

The hydrophobic nature of HhNp makes studying its biochemistry difficult. Thus, the initial characterization of Hh family members was performed using a recombinant form of Shh (ShhN) that was not lipid-modified, which could mimic many of the biological activities thought to be Shh-dependent (10, 12–14, 24, 25). However, the potency of recombinant ShhN was much less than that of ShhNp, which had been purified from the membranes of cells over expressing full-length Shh (16). Although ShhNp was more potent than ShhN, it did not initially appear to be freely diffusible, a requirement necessary for ShhNp to act as a morphogen (26). We have described a potent freely diffusible form of Shh we called s-ShhNp, which migrates as a complex of ~120 kDa. This form of ShhNp appears to be multimeric, because wt Shh can co-immunoprecipitate with an epitope-tagged form of Shh (27). ShhN is not able to form such multimers, suggesting that multimer formation requires one or both of its lipid modifications. We suggested that s-ShhNp is the physiologically relevant form of Shh, because a gradient of this soluble form of Shh could be detected across tissue expressing endogenous levels of Shh. The exact number of Shh molecules in s-ShhNp is unknown, as is the requirement, if any, of additional factors that may act as intermediates between the various Shh molecules. More recently, it has been suggested that palmitoylation of ShhNp may be required for multimerization (23). However, it has also been proposed that s-ShhNp may actually be a lower activity form ShhNp and that palmitoylation is not required for multimerization (28). Beyond these seemingly opposite analyses of s-ShhNp, a structure-function analysis of s-ShhNp has not been performed.

The crystal structure of human recombinant ShhN protein is very
Multimeric Sonic Hedgehog

In this report are marked by an asterisk or shown as deleted amino acids (ShhΔ1–ShhΔ3). The following Hedgehog sequences were aligned: Rattus norvegicus Sonic Hedgehog-rShh, Mus musculus Sonic Hedgehog-mShh, human Sonic Hedgehog-hShh, Gallus gallus Sonic Hedgehog-gShh, Xenopus laevis Sonic Hedgehog-xShh, Takifugu rubripes Sonic Hedgehog-tShh, human Indian Hedgehog-hIhh, Mus musculus Indian Hedgehog-mIhh, human Desert Hedgehog-dShh, Mus musculus Desert Hedgehog-mDhh, and Drosoptila melanogaster Hedgehog-hHh.

### Table 1: Conservation of the amino-terminal domain of Hedgehog

| Shh          | Sequence                        |
|--------------|---------------------------------|
| rShh         | CGPGRFGG-KRQHP-KKL-PLAVKFQ1PN   |
| mShh         | CGPGRFGG-KRHP-KKL-PLAVKFQ1PN    |
| hShh         | CGPGRFGG-KRHP-KKL-PLAVKFQ1PN    |
| gShh         | CGPGRFGG-KRHP-KKL-PLAVKFQ1PN    |
| xShh         | CGPGRFGG-KRHP-KKL-PLAVKFQ1PN    |
| tShh         | CGPGRGYY-RHP-KKL-PLAVKFQ1PN     |
| hIhh         | CGPGRVQ5SRRPPPK-PLKVLAVKFQ5SN  |
| mIhh         | CGPGRVQ5SRRRP-KP-PLKVLAVKFQ5SN  |
| hDhh         | CGPGRPGVQ5RYARKQPLVQLYPFPVG    |
| mDhh         | CGPGRPGVQ5RYARKQPLVQLYPFPVG    |
| Hh           | CGPGRGLG-RHR-A-RLNYLVLQYTF1PN  |
| Δ1           | CG----------------QHP-KKL-PLAVKFQ1PN |
| Δ2           | CG----------------LYAK-PLAVKFQ1PN |
| Δ3           | CG----------------N            |

Similar to that of mouse ShhN (29, 30). However, the structure of human ShhN differs from that of mouse ShhN in one significant way; the structure of a highly conserved amino-terminal domain was visible in human ShhN. This amino-terminal domain of ShhN is among the most highly conserved regions of Hh family members, exhibiting >90% conservation across various homologs (Table 1). Of particular relevance to this work, the amino terminus of ShhN extended ~30 Å away from the rest of the more globular molecule, where it appeared to be stabilized by interactions with adjacent symmetry related molecules. Although it has been suggested that the palmitoylation of ShhNp is required to form s-ShhNp, the human ShhN in this crystal structure was not palmitoylated. To test the hypothesis that the Shh-Shh crystal contacts observed in the human ShhN structure mimic those present in s-ShhNp, we mutated amino acids in this amino-terminal domain of Shh to perform a structure function analyses of s-ShhNp. Here, we provide evidence that large amounts of s-ShhNp can be produced from a stable cell line expressing full-length Shh under the control of an ecycdysone-inducible promoter. This s-ShhNp is multimeric, highly active, and stable, remaining multimeric in the presence of high concentrations of salt and detergent. Underlying this stability of s-ShhNp, we show that a number of protein-protein interactions, as well as protein-lipid interactions are required for the formation of s-ShhNp. Furthermore, our results provide the first evidence based on the structure of Shh for the existence of Shh multimers, supporting the biological relevance of s-ShhNp.

### Experimental Procedures

**Construction of Shh Mutants**—A cDNA expressing full-length rat Shh (Vhh-1), and various Shh mutations, were inserted into a pMT21 vector (11). The various point mutations of Shh were made by altering the correct sequence of the various Shh mutants was verified by DNA sequencing. For the stability and activity of s-ShhNp under a variety of conditions, Shh mutants were analyzed directly by gel filtration to determine its multimeric state (see below). All activity assays were done in duplicate, and each experiment was repeated a minimum of two times. The activity data for all the replicates is presented as mean ± S.D.

**Sizing Analysis of Shh Conditioned Media**—Conditioned media was collected and centrifuged at 4000 × g for 60 min. The cleared media was then concentrated using a Centricon Plus-20. This concentrated media was then centrifuged at 14,000 × g for 10 min and loaded onto a calibrated Superose-12 column, as previously described (27). The molecular mass standards used to calibrate the Superose-12 column were catenase, 232 kDa; aldolase, 158 kDa; albumin, 67 kDa, ovalbumin, 43 kDa; chymotrypsin, 25 kDa; and RNase A, 13.7 kDa. The column was equilibrated in phosphate-buffered saline with 0.1% Nonidet P-40. Every two fractions were pooled and immunoprecipitated using the Shh monoclonal antibody 5E1, followed by immunoblotting with the Shh polyclonal antibody H-160 (Santa Cruz Biotechnology). There is a strong correlation between the ability of the 5E1 monoclonal antibody to recognize a Shh mutant and the ability of that mutant to bind to Ptc (32). Because all the mutants analyzed here were recognized by the 5E1 monoclonal antibody, we assume that their overall structure is intact.

Prior to bioassay analyses of the Superose-12 fractions, Shh-Light2 cells were treated with serial dilutions of detergent in the presence of conditioned media to determine their ability to respond to Shh in the presence of detergent. After establishing that Shh-Light2 cells are unaffected by Nonidet P-40 concentrations <0.006%, we performed sizing analysis of wt Shh conditioned media on a Superose-12 column equilibrated in DMEM and 0.01% Nonidet P-40. Every two fractions were pooled and diluted 1:1 with fresh DMEM to yield a 0.005% final concentration of Nonidet P-40, then assayed for Shh activity using Shh-Light2 cells as previously described (33).

### RESULTS

**Stability and Activity of s-ShhNp**—Beyond being multimeric, very little characterization of s-ShhNp has been described. Here, we determine the stability and activity of s-ShhNp under a variety of conditions,
information that is required prior to purifying this freely diffusible form of ShhNp to homogeneity. In particular, we characterize a cell line that has been engineered to produce large amounts of ShhNp under the control of an inducible ecdysone promoter (Shhl cells), as such a cell line will be a convenient source of s-ShhNp (Fig. 1). Shhl cells were treated with various concentrations of the synthetic ecdysone derivative Ponasterone A to activate Shh expression. Conditioned medium from these cells was then isolated and used to examine the presence and activity of Shh. Active Shh was produced in cell media in a dose-dependent manner, up to 5 μg/ml Ponasterone A (Fig. 1A). Although Shh was expressed in the absence of Ponasterone A, its addition increased ShhNp levels in conditioned media ~10-fold. The ShhNp in the conditioned media was active (Fig. 1B), showing dose-dependent activation of, a cell line stably expressing an Shh-responsive promoter driving luciferase expression (Shh-Light2 cells) (33). We fractionated the conditioned media from Shhl cells on a Superose-12 column, to verify that the conditioned media contained s-ShhNp. Every two Superose-12 column fractions were pooled, immunoprecipitated using the Shh monoclonal antibody 5E1, resolved by SDS-PAGE, and immunoblotted for Shh. ShhNp from the conditioned media of Shhl cells migrated in a similar sized peak to that previously described for s-ShhNp, with the majority of Shh migrating as a multimer at fraction #31 (Fig. 1C), with a molecular size of ~120 kDa. A minor peak of ShhNp was also observed in fraction #41, migrating with a molecular size consistent with it being monomeric. The ratio of multimeric to monomeric ShhNp produced in the conditioned medium by Shhl cells was ~11:1.

We next tested the ability of s-ShhNp to withstand high salt or high detergent concentrations, to determine the stability of s-ShhNp under standard purification conditions. For the high salt experiment, both the Shhl-conditioned media and the Superose-12 column running buffer were adjusted to 0.5 M NaCl. Under these conditions, s-ShhNp migrated through the Superose-12 column in a manner similar to that of s-ShhNp fractionated under isotonic conditions, with a major peak at fraction #30 and a minor monomeric peak at fraction #40 (Fig. 2A). To test the stability of s-ShhNp in a non-ionic detergent we performed sizing analyses of Shhl-conditioned media in the presence of 1% Nonidet P-40. Under these conditions, the majority of Shh in the conditioned media still migrated as a multimer, with the s-ShhNp peak appearing slightly larger than s-ShhNp fractionated under low detergent conditions (Fig. 2B). Thus, Shhl cells produce large amount of s-ShhNp in a form that is very stable to both high concentrations of salt and detergent.

Although we have previously shown that ShhNp in conditioned media is active, it remains unclear as to whether this activity corresponds to s-ShhNp or whether the monomeric form of ShhNp present in the conditioned media is the sole contributor of this activity. To resolve this issue, we took conditioned media from the Shhl cells and fractionated it over a Superose-12 column equilibrated with a running buffer consisting of DMEM with reduced Nonidet P-40 (0.01%). The fractions were then diluted with an equal volume of DMEM containing no detergent, for a final Nonidet P-40 concentration of 0.005%, and added to Shh-Light2 cells to assay for Shh activity. Under these conditions, the peak of Shh activity isolated from the Superose-12 column fractions migrated in a manner that correlated well with Shh immunoreactivity, with a large peak around fraction #30 and a smaller peak around fraction #42 (Fig. 2C). Thus, the activity of ShhNp found in conditioned media resides predominantly in the Shh multimer, demonstrating that s-ShhNp represents the majority of activity in Shh conditioned media.

Analysis of Shh Palmitoylation Site Mutants—Two recent reports have provided conflicting results on the role palmitoylation plays in s-ShhNp formation (23, 28). As we intended to analyze the role the amino-terminal domain of Shh plays in its multimerization, and this encompasses the site of palmitoylation, we began our structure-function analyses of s-ShhNp by examining mutations at the palmitate acceptor site (Cys-25), ShhC25S and ShhC25A. Both of these mutants were processed to their faster migrating cholesterol modified forms in a...
Multimeric Sonic Hedgehog

manner comparable to wt Shh (Fig. 3A). These mutants were also more highly expressed in conditioned media than wt Shh, consistent with palmitoylation playing a role in tethering Shh to cellular membranes. Furthermore, conditioned media from cells expressing these two Shh mutants had significantly less activity than that of wt Shh (Fig. 3B), consistent with palmitoylation of Cys-25 playing an important role in Shh activity. Sizing analysis of ShhC25S and ShhC25A conditioned media showed that these two proteins migrate in a manner comparable to ShhN, predominantly as a monomeric form at fraction 41 (Fig. 3C). These results are consistent with palmitoylation of Shh playing an important role in the formation of s-ShhNp.

Analysis of Shh Crystal Contact Mutations—In the crystal structure of ShhN (Fig. 4), specific amino acids that appeared to coordinate the intermolecular interaction of adjacent Shh molecules could be identified (30). These amino acids include Pro-27, Phe-31, and Phe-48 on its amino-terminal domain as well as a number of amino acids surrounding the Zn$^{2+}$-binding domain. To determine the role these amino acids play in Shh multimerization and activity we made non-conservative mutations in Shh, ShhP27T, ShhF31H, and ShhF48H, to alter the properties of the amino acid at that position. Plasmids expressing these Shh mutants, or wt Shh, were transfected into HEK cells, and the conditioned media were examined for Shh expression, activity, or multimeric state (Fig. 5). We also examined the lysates from these cells, which showed that all the mutant proteins expressed to similar levels as wt Shh, for both full-length (u-Shh) and processed forms of Shh (ShhNp). The amount of Shh in conditioned media of ShhP27T, ShhF31H, or ShhF48H transfected cells was comparable to that of wild-type Shh transfected cells. The activity of these Shh point mutants did not correlate well with Shh protein levels. ShhP27T had decreased activity relative to wt Shh, having activity similar to that of media from vector transfected cells, while ShhF31H and ShhF48H had activity that on average was comparable to that of wt Shh, demonstrating that these latter two mutations did not have significant effects on the processing, secretion, or activity of Shh. Based on these results we made an additional mutation between ShhP27T and ShhF31H, mutating the highly conserved Gly-30 to an Arg (ShhG30R). Interestingly, given the location of this conserved amino acid, this Shh mutant behaved differently from our crystal contact mutations, as the vast majority of ShhG30R did not appear to efficiently process from its full-length form to ShhNp. Consequently, little ShhNp was found in the conditioned media of cells expressing ShhG30R. We next fractionated conditioned media from cells expressing the various Shh mutants over a Superose-12 column, to directly address the ability of these Shh intermolecular crystal contact site mutants to form s-ShhNp. The ability of ShhP27T, ShhF31H, and ShhF48H to form multimers was disrupted, relative to wt Shh. The ratio of multimeric to monomeric ShhNp is 9:1 for wt Shh, whereas it is much less for these mutants (Fig. 5C). We were unable to obtain enough ShhG30R in the conditioned media of cells expressing it to obtain a reliable estimate of its molecular size. The inability of these latter Shh mutants to form s-ShhNp was consistently observed. However, we observed more variability in the size of the resulting product (data not shown), ranging, for any particular mutant, from an intermediate sized ShhNp complex to monomeric ShhNp. We do not know the reason for this variability, but suspect that it reflects the decreased ability of these mutants to form stable Shh multimers. These results are consistent with the proposed role the amino-terminal domain of Shh plays in activity and multimerization.

In the human ShhN crystal structure various residues on the amino-terminal domain of one Shh molecule contact a central region of an adjacent ShhN molecule (see Fig. 4B). Based on this structure, we made three Shh “pocket” mutants in this central region of Shh, which we predicted would disrupt these intermolecular interactions, ShhL140R, ShhW173E, and ShhH183A. Plasmids expressing these pocket mutations, or wt Shh, were transfected into HEK cells, and Shh expression was analyzed in both the cell lysate and the conditioned media of the transfected cells. Unfortunately, the vast majority of the Shh pocket mutants did not process efficiently from full-length Shh into ShhNp

FIGURE 3. s-ShhNp formation requires palmitoylation. The lysates of HEK cells expressing wt Shh, ShhC25S, ShhC25A or a control vector, were analyzed by immunoblotting (A, bottom panel), assayed for its ability to activate a Hh-dependent reporter assay (B), or fractionated over a gel-filtration column to determine the presence of s-ShhNp. The fractions in which molecular mass standards (kDa) elute are marked (C).

FIGURE 4. Structural analyses of Shh-Shh interactions. Intermolecular interactions observed in the human Shh-N crystal structure. A, surface representation of two molecules, one shown in green and one in blue. The darker blue region shows the extended chain containing the amino-terminal amino acids 25–45, which wrap around the symmetry related molecule in the crystal, forming a number of close contacts. B, a close up view highlighting several of the residues mutated in this study. Orange residues include Cys-25, Pro-27, and Phe-31 from one molecule, corresponding to the dark blue region in panel A. Residues shown in red (Leu-140, Trp-173, and His-183) are from the adjacent green molecule. These models were made using the program PyMOL version 0.98 (36).
HEK cells were transfected with plasmids expressing either wt Shh, ShhP27T, ShhG30R, ShhF31H, or a control vector were analyzed by immunoblotting for the expression of full-length Shh (u-Shh), the processed form of Shh (ShhNp), or tubulin (Tub) (A, top panel). Shh present in the conditioned media of these cells was analyzed by immunoblotting (A, bottom panel), assayed for its ability to activate a Hh-dependent reporter assay (B), or fractionated over a gel-filtration column to determine the presence of s-ShhNp. The fractions in which molecular mass standards (kDa) elute are marked (B). On average, the expression of wt Shh, ShhP27T, ShhF31H, and ShhF48H in the conditioned medium was comparable, unlike that observed in the individual experiment shown.

**FIGURE 5.** Identification of amino acids important for s-ShhNp formation. The lysates of HEK cells expressing wt Shh, ShhP27T, ShhG30R, ShhF31H, ShhF48H, or a control vector were analyzed by immunoblotting for the expression of full-length Shh (u-Shh), the processed form of Shh (ShhNp), or tubulin (Tub) (A, top panel). Shh present in the conditioned media of these cells was analyzed by immunoblotting (A, bottom panel), assayed for its ability to activate a Hh-dependent reporter assay (B), or fractionated over a gel-filtration column to determine the presence of s-ShhNp. The fractions in which molecular mass standards (kDa) elute are marked (C). On average, the expression of wt Shh, ShhP27T, ShhF31H, and ShhF48H in the conditioned medium was comparable, unlike that observed in the individual experiment shown.

**FIGURE 6.** Analysis of Shh binding pocket mutations. The lysates of HEK cells expressing wt Shh, ShhL140R, ShhW173E, ShhH183A, or a control vector were analyzed by immunoblotting for the expression of full-length Shh (u-Shh), the processed form of Shh (ShhNp), or tubulin (Tub) (A, top panel). Shh present in the conditioned media of these cells was analyzed by immunoblotting (A, bottom panel) or assayed for its ability to activate a Hh-dependent reporter assay (B).

(Fig. 6A). Consistent with this observation, only small amounts of ShhNp were found in the conditioned media of cells transfected with these mutants, which consequently exhibited lower activity. Like ShhG30R, the pocket mutants do not produce sufficient ShhNp in the media to allow analysis by gel filtration.

**Analysis of Shh Amino-terminal Truncations**—Although the crystal structure of ShhN indicates that amino-terminal amino acids of Shh are involved in forming intermolecular contacts that stabilize s-ShhNp, we did not identify a single amino acid absolutely required for this interaction. Therefore, we made a series of amino-terminal deletions to ascertain whether these Shh truncations would have more dramatic effects on s-ShhNp formation. When designing these deletion mutants, we began our truncations at Pro-27 to avoid disrupting the palmitoylation of Shh, which plays an important role in Shh multimerization. Furthermore, we now provide evidence, based on intermolecular interactions observed in the crystal structure of human ShhN (30), that s-ShhNp formation requires the presence of its highly conserved amino-terminal domain. Thus, we provide, for the first time, evidence for the existence of s-ShhNp based on the structural analysis of ShhΔ1, ShhΔ2, or ShhΔ3, and Shh expression in both cell lysates and conditioned media was analyzed (Fig. 7A). Wt Shh, ShhΔ1, and ShhΔ2 were processed normally in the cell lysate, as both full-length and cholesterol modified Shh were observed. ShhΔ1 and ShhΔ2 were also detectable in the conditioned media of the various transfected cells, in amounts comparable to wt Shh. The ShhΔ3 mutant was also expressed as its full-length unprocessed form but did not get processed to its cholesterol modified form. Consistent with this lack of processing, little ShhΔ3 was found in the conditioned media of ShhΔ3-transfected cells. Although conditioned medium from cells expressing wt Shh activated the Shh-Light2 cells ~5-fold compared with control medium, the conditioned media from cells transfected with the various Shh amino-terminal truncations had little activity (Fig. 7B). We next performed sizing analysis on the conditioned media from cells transfected with ShhΔ1 or ShhΔ2. The ability of ShhΔ1 to form multimers was compromised relative to wt, with >50% decrease in its ability to form s-ShhNp. The ability of ShhΔ2 to form multimers was more compromised than ShhΔ1, because ShhΔ2 always appeared to be monomeric. These results are consistent with the highly conserved amino-terminal region of Shh playing an important role in Shh multimerization.

**DISCUSSION**

Here, we show that large amounts of ShhNp are produced from Shh cells, providing a convenient source of material for the purification and biochemical analysis of s-ShhNp. Although Shh in conditioned media exists as either a multimeric form, s-ShhNp, or as a less abundant monomeric form, we show that the bulk of Shh activity migrates with s-ShhNp. The s-ShhNp complex is tightly associated, as high concentrations of either NaCl or Nonidet P-40 have little effect on its multimeric state, suggesting that multiple types of interactions are required to maintain this stability. Consistent with this suggestion, we show that s-ShhNp formation requires palmitoylation of ShhNp. Furthermore, we now provide evidence, based on intermolecular interactions observed in the crystal structure of human ShhN (30), that s-ShhNp formation requires the presence of its highly conserved amino-terminal domain. Thus, we provide, for the first time, evidence for the existence of s-ShhNp based on the structural analysis of
Multimeric Sonic Hedgehog

### TABLE 2
Summary of s-ShhNp structure-function analyses

Various Shh mutants, or wt Shh, were analyzed for their ability to process into their cholesterol-modified form, become multimeric, or have biological activity. This table summarizes the results presented in this report. We assigned each analyses a numerical value of ++++ (highest) to − (lowest) in comparison to wild-type Shh. For the processing and multimeric state results, this number is based on the average of the replicates of each analyses. When any particular analysis was not performed an “ND” was inserted in the appropriate column.

| Mutant  | Type of mutation | Processed | Multimeric state | Activity | Apparent molecular mass of peaks |
|---------|------------------|-----------|------------------|----------|----------------------------------|
|         |                  |           |                  |          |                                  |
| ShhΔ1   | None             | ++++      | ++++             | ++++     | 155                              |
| ShhΔ2   | Truncation, aa 27–34 | ++++      | +                | ++++     | 155                              |
| ShhΔ3   | Truncation, aa 27–42 | ++++      | −                | −        | 155                              |
| ShhP27T | Crystal contact  | −         | ND*              | −        | 155                              |
| ShhL140R| Highly conserved | +         | ND               | −        | 155                              |
| ShhF31H | Crystal contact  | ++++      | +                | ++++     | 155                              |
| ShhF48H | Crystal contact  | ++++      | ++++             | ++++     | 155                              |
| ShhL140R| Binding pocket   | +         | −                | −        | 155                              |
| ShhW173E| Binding pocket   | +/−       | −                | −        | 155                              |
| ShhH183A| Binding pocket   | −         | −                | −        | 155                              |
| ShhC25S| Palmitoylation site | ++++      | −                | −        | 155                              |
| ShhC25A| Palmitoylation site | ++++      | −                | −        | 155                              |

* ND, not determined.

human ShhN. Combined, our results show that both protein–protein and protein–lipid interactions are required for s-ShhNp formation.

Our analyses show that at least three regions of ShhNp are required for s-ShhNp formation, amino acids 27–34, amino acids 35–48, and the palmitoylation acceptor site Cys-25. These first two regions of Shh were predicted to be involved in Shh-Shh interactions based on the crystal structure of ShhN (30). This structure shows that Pro-27 and Phe-31 make extensive hydrophobic contacts with a second Shh molecule at Phe-48, Trp-173, and His-183 (see Fig. 4B). Non-conservative mutation of either Pro-27 or Phe-31, or removal of amino acids 27–34, renders ShhNp less able to form s-ShhNp. Although the ability of all three of these Shh mutants to form s-ShhNp is equally reduced, they differ in what happens to the portion of ShhNp unable to form s-ShhNp. Rather than being completely converted into monomeric ShhNp, ShhP27T and ShhF31H appear to also form an intermediate sized ShhNp complex. Conversely, ShhΔ1 that cannot form s-ShhNp converts completely into its monomeric form. These results are consistent with loss of amino acids 27–34 having a more dramatic affect on s-ShhNp formation than mutation of either Pro-27 or Phe-31, as might be expected given that Pro-27 and Phe-31 are both missing in ShhΔ1. ShhΔ2, which encompasses a loss of amino acids 27–42, is almost completely monomeric. Because ShhΔ2 is less multimeric than ShhΔ1 (Table 2), we conclude that amino acids 35–42 also play an important structural role in s-ShhNp formation. A region of Shh within amino acids 27–42 that may contribute to s-ShhNp formation is the Cardin-Weintraub protein–heparin interaction domain (34). Mutation of amino acids within this highly conserved motif eliminates the high affinity interaction of ShhN with various heparin sulfate proteoglycans. The biological activity of these Shh mutants is reduced in some bioassays but is comparable to wt ShhN in other assays, suggesting that Shh-heparin sulfate proteoglycan interactions are important only for some specific subset of Shh activity. It has been previously shown that FGF is able to form dimers that are stabilized through the interactions of each monomer with a common heparin sulfate proteoglycan (35). Therefore, we speculate that the Cardin-Weintraub motif might also play a role in stabilizing Shh-Shh interactions, through association with a heparin sulfate proteoglycan. Although we have presented evidence for two classes of mutations that affect the formation of s-ShhNp, those that affect various intermolecular Shh-Shh interactions and those that affect the site of palmitoylation, it remains possible that both classes of mutations affect a common function. For example, if palmitoylation of Shh requires it to be multimeric, then disrupting Shh-Shh interactions would lead to a loss of palmitoylation. In this case, the multimeric state of the various Shh mutants tested would correlate with their level of palmitoylation. However, analyses of the palmitoylation state of Shh mutants do not correlate with their multimerization state (data not shown).

It was previously suggested that palmitoylation of ShhNp is important for s-ShhNp formation, a conclusion that has remained controversial for a number of reasons (23). Firstly, the ratio of ShhNp multimer to ShhNp monomer shown in the previous report, using wt Shh, was low, with the vast majority of ShhNp appearing monomeric. When the ShhC25S mutant was analyzed the minor multimeric peak did not appear to form, suggesting that palmitoylation was necessary for s-ShhNp formation. However, because of the low multimer-monomer ratio observed with wt Shh, it remained possible that the ShhC25S mutation had only a minor effect on multimerization and that this small difference was lost in their low multimer:monomer ratio. Secondly, their conclusion was based solely on the analysis of a Shh mutant in which the palmitate acceptor Cys-25 was converted into a Ser, a mutation that appears to also affect some function of Shh not related to palmitoylation (22). When the activity of recombinant ShhN or Shh-N25S purified from bacteria are compared the potency of wt ShhN can be 10-fold greater than that of Shh-N25S. Because recombinant ShhN is not palmitoylated, the reduced activity of the Shh-N25S mutant must be due to the Cys to Ser substitution disrupting some structural role that the amino terminus of Shh normally plays. A similar argument can be made when analyzing the ShhC25S mutant expressed in eukaryotes, that the ShhC25S mutation disrupts the structure in some way, affecting its ability to form multimers. Finally, the opposite conclusion, that palmitoylation of ShhNp is not important for s-ShhNp formation, was reached by another group using a similar approach (28). In this second report, the majority of ShhNp in the conditioned media appears to be multimeric, as does a ShhC25S mutant. The results presented in this latter report were particularly convincing because of the quality of the sizing data presented, which showed a high ShhNp multimer:monomer ratio in conditioned media. Our results clearly show that palmitoylation of ShhNp is required for s-ShhNp formation. We present results showing that the majority of wt ShhNp in conditioned media is present in a multimeric form, with only a minor form of ShhNp migrating as a monomer. Under conditions in which the ratio of multimer to monomer is very high, ShhC25S is almost entirely monomeric. Moreover, we also analyzed a ShhC25A mutant for its ability to form multimers and found that this mutant was also unable to form s-ShhNp, migrating predominantly as a monomer. This mutation should have minimal effects on the overall structure and activity of Shh (22). Therefore, we conclude that palmitoylation of ShhNp is important for s-ShhNp formation. The
differences between our methodology and that of others (28) are sufficiently large that it is difficult to reconcile the differences in our conclusions. However, one major difference between the two analyses is the type of cells used to produce s-ShhNp. Therefore, it remains possible that some cell types will have the ability to form s-ShhNp in a manner independent of palmitoylation.

When ShhC25S is inserted into the wt mouse Shh loci, such that it is expressed in a manner analogous to wt Shh, the resulting mice exhibit phenotypes consistent with defects in long range signaling (23). Our work, along with that of the previous report, suggests that formation of s-ShhNp requires palmitoylation of ShhNp. Consequently, these results suggest that the function of s-ShhNp is to act directly in long range Shh signaling. Our work is consistent with this proposal, as we clearly show that the bulk of ShhNp activity in conditioned media is potent, multimeric and stably associated, three properties that would be required for the long range signaling form of Shh. Furthermore, we are able to separate the multimeric state of s-ShhNp from its biological activity. For example, while the potency of ShhΔI is dramatically reduced, it is still capable of forming s-ShhNp. Conversely, we are also able to produce mutant forms of ShhNp, such as ShhF31H, that are as potent as wt s-ShhN but are not as multimeric. This low correlation between potency and multimerization state of s-ShhNp suggests that the purpose of ShhN multimerization is not solely to increase its activity. Thus, our results are not consistent with the function of s-ShhNp being to regulate its activity, in either a positive or a negative fashion, but rather suggest that the function of s-ShhNp is to keep ShhNp in a form capable of moving through the extracellular environment.

Based on the crystal structure of recombinant human ShhN (30), we identified amino-terminal regions of Shh important for s-ShhNp formation. Furthermore, we also showed that palmitoylation of ShhNp was important for its multimerization. These conclusions appear inconsistent with previous biochemical analysis of ShhN that showed that ShhN was monomeric and that recombinant ShhNp was not palmitoylated (27, 28). We speculate that under the high protein concentrations normally required for protein crystallization ShhN may spontaneously form multimers, bypassing the normal physiological requirement for lipid mediated stabilization of s-ShhNp. However in vivo, where Shh concentrations are much lower, palmitoylation of ShhNp would be required to stabilize the protein-protein interactions within the Shh multimer. Moreover, the structure of human ShhN previously reported was not that of wt ShhN but rather was that of ShhN-C25II, which was originally engineered to mimic the palmitate that normally modifies Cys-25 (30). Although ShhN-C25II has less activity than palmitoylated ShhN, it may, under the high protein concentrations present in the ShhN-C25II crystal, faithfully mimic the ability of the palmitate to stabilize the Shh intermolecular interactions found in s-ShhNp. We have taken advantage of this structure to provide here the first structural evidence that supports the existence of a multimeric form of ShhNp, s-ShhNp.

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