Mapping Sonic Hedgehog-Receptor Interactions by Steric Interference*

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We have identified regions in the Sonic hedgehog (Shh) molecule that are important for Patched (Ptc) receptor binding by targeting selected surface amino acid residues with probes of diverse sizes and shapes and assessing the effects of these modifications on function. Eleven amino acid residues that surround the surface of the protein were chosen for these studies and mutated to cysteine residues. These cysteines were then selectively modified with thiol-specific probes, and the modified proteins were tested for hedgehog receptor binding activity and their ability to induce differentiation of C3H10T1/2 cells into osteoblasts. Based on these analyses, approximately one-third of the Shh surface can be modified without effect on function regardless of the size of the attachment. These sites are located near to where the C terminus protrudes from the surface of the protein. All other sites were sensitive to modification, indicating that the interaction of Shh with its primary receptor Ptc is mediated over a large surface of the Shh protein. For sites Asn-50 and Ser-156, function was lost with the smallest of the probes tested, indicating that these residues are in close proximity to the Ptc-binding site. The epitope for the neutralizing mAb 5E1 mapped to a close but distinct region of the structure. The structure-activity data provide a unique view of the interactions between Shh and Ptc that is not readily attainable by conventional mapping strategies.

Hedgehog proteins are a highly conserved family of extracellular signaling molecules with fundamental roles in embryonic development both in vertebrates and in invertebrates (for reviews see Refs. 1–4). In mammals, three homologs have been identified that are referred to as Sonic, Indian, and Desert hedgehog. Of these forms, Sonic hedgehog (Shh) has been the most extensively characterized. Shh is involved in diverse embryonic events, including the induction of floor plate, the establishment of ventral polarity within the central nervous system, and proper anterior-posterior patterning of the developing embryo (5–8). In mediating these effects, Shh is believed to act both as a short range, contact-dependent inducer and as a long range morphogen (2, 7–11). It is unclear whether the same molecule phosphatase; PEG, polyethylene glycol; PAGE, polyacrylamide gel electrophoresis; mAb, monoclonal antibody.

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1. The abbreviations used are: Shh, Sonic hedgehog; Ptc, Patched; Hh, hedgehog; MES, 4-morpholineethanesulfonic acid; DTT, dithiothreitol; BzM, benzophenone-4-maleimide; NEM, N-ethylmaleimide; AP, alkaline phosphatase; PEG, polyethylene glycol; PAGE, polyacrylamide gel electrophoresis; mAb, monoclonal antibody.

importance of Shh for correct embryonic development is highlighted by the severity of malformations associated with human mutations in the Shh gene (12) and by the developmental defects observed in the Shh knockout mouse leading ultimately to its embryonic lethal phenotype (13).

Shh is synthesized as a 45-kDa precursor protein that is cleaved autocatalytically to yield a 20-kDa N-terminal fragment (residues 24–197 in the human gene sequence) that is responsible for all known hedgehog biological activity and a 25-kDa C-terminal fragment that contains the auto-processing machinery (14–16). The N-terminal fragment remains membrane-associated through the addition of two lipid tethers, a palmitic acid at its N terminus (17) and a cholesterol at its C terminus (18, 19). In vivo, the lipid tethers restrict the tissue localization of the Hedgehog signal and presumably have evolved as part of the mechanism for regulating short range-long range signaling. Whereas both modifications are likely to be important for tethering Shh to the plasma membrane, lipid modifications on the N terminus result in about a 30-fold increase in potency (17). How the N-terminal modification regulates potency is unknown. The three-dimensional crystal structure of a fragment of murine Shh (residues 39–195) has been solved (20). This information has proved to be invaluable for probing structure-function relationships of the protein.

Although the mechanism of action of hedgehog proteins is not fully understood, biochemical and genetic data suggest that the receptor for Shh is the product of the tumor suppressor gene patched (ptc) (21–23) and that other proteins, including smoothened (smo) (23, 24), Cubitus interruptus, or its mammalian counterpart ghi (25, 26) and fused (27), are involved in the hedgehog signaling pathway. Homology between the ptc sequence and other gene products suggests that it is a member of the 12 transmembrane family of proteins. Although Ptc is the receptor for Shh, it also acts as a negative modulator of the Shh signal. In the absence of Shh, Ptc represses signaling through Smo. Shh binding to Ptc causes derepression of Smo, allowing signaling to occur. Subsequent induction of Ptc may act to turn off hedgehog (Hh) signaling. From the analysis of smo null mutants in Drosophila, Ptc appears to bind Hh without the participation of Smo (28). Other Hh-binding proteins, including a ptc homolog, ptc-2 (23), and hedgehog-interacting protein (29) have been identified, raising the potential for additional levels of regulation. In order to study the interactions of Shh with Ptc, we developed a mapping strategy where selected amino acid residues on the surface of Shh were mutated into cysteine residues, and after modification of these cysteines with probes of different size and shape, we determined which of the modifications disrupted function. The structure-activity data provide a unique view of Shh-Ptc interactions. This approach has significant advantages over most conventional mapping strategies and should be widely applicable to most other proteins, particularly those for which a structure is available.
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EXPERIMENTAL PROCEDURES

Construction of Shh Mutants—Shh (residues 24–197 in the human sequence) and Shh mutants were expressed in Escherichia coli as His-tagged fusion proteins as described previously (17, 30). Point mutations were incorporated into the Shh DNA by unique site elimination mutagenesis using an Amersham Pharmacia Biotech kit following the manufacturer’s recommended protocol. Mutagenic oligonucleotides were used such that the incorporation of each mutation also resulted in the addition or removal of a specific restriction enzyme site. The following mutagenic primers were used, and the resulting restriction sites that were added as a result of the mutagenesis steps are listed below: 1) A192C, 5’-GAG TCA TGC GCC CGA TTT TGC GAC CAG GTA CTC TGC TTT CAC CTC TGA GTC 3’; 2) N69C, 5’-GAGTTC CTT AAA TCG CTC GGA GCA CCT GGA GAT CTT CTC TTT CAC CTC TGA GTC 3’; 3) Bsp 121I site; 4) Y80C, 5’-CAT CTT TAA ATA TGA TGT CCG GTG TGC AAAT TGG GGG GTA GCT TTA AGG TCT GTA GTC 3’; 5) N91C, 5’-CAT CAG CCT GTC GGG TAG CTC TTT CTT CTT GGT CTT CTC AGC CAC ACA GGG GAT GTC 3’, which introduces a new MsiI site; 6) K105C, 5’-GAG ATG GCG AAA GGG TTT AAG CAG CTT TCA CAC CTC TGA GTC 3’, which introduces a new NheI site; 7) N115C, 5’-GTT TCA TTC TCA GGA ACT GAC ACA TCA CCG TCC CGA TTT TGC GCA CAC TGA CAC CCA GTC GAA GCA GGC CTC CAC CGC CAG GC 3’; 8) S135C, 5’-GTA GTC GAG CAG ACA TGT CCA CCG TCC CGA TTT TGC GCA CAC CTC TGA GTC 3’, which introduces a new PvuII site; 9) S156C, 5’-GCA GGA CCT GCT CGT ACT CTT GAT GCA GCC GGC GGT CTT CTC AGC CAC ACA GGG GAT GTC 3’, which introduces a new NciI site; 10) S177C, 5’-CAG TTA ATG TGT CCG GTG TGC AAAT TGG GGG GTA GCT TTA AGG TCT GTA GTC 3’, which removes a Hinfl site; and 11) G196C, 5’-GTA CAC CCA GTC GAA GCA GGC CTC CAC CCG CAG GC 3’, which removes an MspI site. The fidelity of the resulting constructs was verified by DNA sequencing. Expression vectors were generated by subcloning the DNA inserts into phosphatase-treated, 5.64-kilobase pET11d vector backbone of p6H-Shh (30), which encodes a 6-histidine tag and an enterokinase cleavage site engineered immediately at the start of the mature Shh sequence. The presence of the introduced restriction site change was reconfirmed in the expression vector. The expression vectors were transformed into competent E. coli BL21(DE3)pLysS, colonies were selected, induced with isopropyl-1-thio-β-D-galactopyranoside, and screened for Shh expression as described previously (17, 30). Shh DNA containing C24II and A192II mutations was used as the template for mutagenesis steps 2–12. The C24II framework, in which cysteine 24 was replaced with two isoleucine residues, was selected for these studies since we had determined that modifications on Cys-24 would otherwise compromise the mapping analysis. The C24II substitution not only prevented the formation of unwanted posttranslational products but generated a form of the protein that was 10 times as active as wild type Shh in the C3H10T1/2 differentiation assay (2).

Purification and Analysis of Shh Mutants—The mutant Shh proteins were purified following the strategy previously described for the purification of wild type Shh (30). Bacteria expressing the mutants were lysed in a French press, and particulates were removed by centrifugation. The clarified lysates were subjected to sequential chromatography of wild type Shh (30). Purification and titration with serial 2-fold dilutions of each test compound in the presence of 5 mM dithiothreitol (DTT) were incubated with Ptc-transfected embryonic kidney 293 cells (30) for 1 h at room temperature and subjected to cross-linking and analysis as described previously (31). Western blots were probed first with rabbit anti-Shh antibody and then with affinity purified rabbit anti-Ptc antibody (30). Ptc binding and C3H10T1/2 AP induction assays were performed as described previously (30). For Ptc binding experiments, EBNA 293 cells transiently transfected with a myc-tagged, C-terminally truncated murine ptc construct (22) (a gift of Matt Scott, Stanford University) were titrated with serial 2-fold dilutions of each test compound in the presence of 5 mM of the reporter. Cells were washed, fixed, and read on a fluorescence-activated cell sorter. Binding constants were calculated from single determinations for each sample. For assessing activity, C3H10T1/2 cells (American Type Culture Collection) were incubated for 5 days with serial dilutions of each Shh preparation. The cells were lysed and assayed for alkaline phosphatase (AP) activity using the chromogenic substrate p-nitrophenyl phosphate and read at 405 nm. Each sample was analyzed in duplicate, and EC50 values were measured from the mean data values.

RESULTS AND DISCUSSION

Production and Characterization of Shh Surface Mutants—We have used the x-ray structure of Shh to identify potential surface residues that could serve as targets for modification. Eleven sites were selected for these studies. Most of these sites lie on loops where minimal contacts occur between the side chains of the selected residues and other parts of the molecule, and together they cover the entire surface of the molecule. The positions of the sites with respect to the Shh structure are summarized in Fig. 1. These amino acids were mutated to cysteine residues by unique site elimination mutagenesis, and the mutated genes were expressed in E. coli.

For cross-linking studies with BzM, samples of Shh at 1.1 mg/ml in 50 mM MES, pH 6.5, 230 mM NaCl, 0.13 mM DTT, 0.1% Tween 80 were treated with a stoichiometric amount of cross-linker (0.5 mM diluted from a 25 mM stock solution). The preformed Shh-BzM conjugates were incubated with Ptc-transfected embryonic kidney 293 cells (30) for 1 h at room temperature and subjected to cross-linking and analysis as described previously (31). Western blots were probed first with rabbit anti-Shh antibody and then with affinity purified rabbit anti-Ptc antibody (30).

Ptc binding and C3H10T1/2 AP induction assays were performed as described previously (30). For Ptc binding experiments, EBNA 293 cells transiently transfected with a myc-tagged, C-terminally truncated murine ptc construct (22) (a gift of Matt Scott, Stanford University) were titrated with serial 2-fold dilutions of each test compound in the presence of 5 mM of the reporter. Cells were washed, fixed, and read on a fluorescence-activated cell sorter. Binding constants were calculated from single determinations for each sample. For assessing activity, C3H10T1/2 cells (American Type Culture Collection) were incubated for 5 days with serial dilutions of each Shh preparation. The cells were lysed and assayed for alkaline phosphatase (AP) activity using the chromogenic substrate p-nitrophenyl phosphate and read at 405 nm. Each sample was analyzed in duplicate, and EC50 values were measured from the mean data values.

2 F. R. Taylor and E. A. Garber, unpublished data.

3 P. A. Boriack-Sjodin and E. A. Garber, unpublished results.
Hedgehog proteins that had been treated with PEG 5000 maleimide, NEM, or PEG maleimide were evaluated for Ptc binding activity on EBNA 293 cells transfected with a truncated form of the gene for Ptc and for their ability to induce differentiation of C3H10T1/2 cells.

| Protein, Shh variant | EC<sub>50</sub> in C3H10T1/2 assay in µg/ml | K<sub>d</sub> for Ptc binding in ng/ml (× 10<sup>-12</sup>) |
|---------------------|------------------------------------------|-----------------------------------------------|
| Unmodified | PEG 5000 | NEM | Unmodified | PEG 5000 | NEM |
| Wild type | 2 | >20 | 1 | 4 | 84 | 2 |
| C24II/A192C | 0.3 | 0.3 | 2 | 2 | 1 |
| +N69C | 0.4 | 4 | 0.3 | 2 | 64 | 1 |
| +Y80C | 0.2 | 0.2 | 0.2 | 4 | 4 | 4 |
| +N91C | 0.3 | 0.2 | 0.2 | 6 | 6 | 2 |
| +K105C<sup>a</sup> | 0.2 | — | — | — | — | — |
| +N115C | 1.5 | >20 | 2 | 8 | 70 | 1 |
| +S135C | 0.4 | 0.6 | 0.2 | 8 | 20 | 2 |
| +S156C | 0.4 | 2 | 0.2 | 8 | 66 | 1 |
| +G169C | 0.3 | 0.5 | 0.3 | 4 | 60 | 4 |
| +S177C | 1 | 5 | 2 | 8 | 170 | 16 |

<sup>a</sup> Modification chemistries failed.

*Fig. 2.* Analysis of pegylated Shh by SDS-PAGE. Shh mutants (4 µg/lane) were subjected to SDS-PAGE on a 10–20% gradient gel (Daiichi) (A) without pegylation and (B) after treatment with 5000 PEG maleimide. Proteins were stained with Coomassie Brilliant Blue. Lane designations correspond to the individual mutant analyzed. The –PEG designation in B is Shh C24II/A197C that had not been treated with PEG. MW Stds are prestained high molecular weight markers. Apparent masses of the molecular weight standards are indicated at the left.

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Typically we observe that modification reactions with maleimides go to about 95% completion. As shown in Fig. 2B, no untreated Shh remained after treatment of the mutants with the PEG 5000 maleimide, but about 5% of the product contained a single modification as evidenced by the presence of the minor band at 32–35 kDa. Throughout the studies described below, assays were performed on products that were treated with the maleimide probes and quenched to prevent further reaction but that had not been further purified. Consequently, a complete blockage of function after modification would be expected to decrease binding or potency by about 20-fold. Our inability to modify cysteine 105 in the K105C mutant was probably due to inadequate exposure of the thiol group on the engineered cysteine. After denaturation and subsequent reaction with PEG, we observed a shift in mass consistent with modification at this site (data not shown).

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The pegylated mutants were tested for function in the Ptc binding and C3H10T1/2 assays. Mutants N69C, Y80C, and...
N91C were fully active in both assays; mutants S135C and G169C were slightly reduced in their activity in the C3H10T1/2 assay but then exhibited a more significant loss of potency for Ptc binding; and mutants N50C, N115C, S156C, and S177C were greatly reduced in activity both in their ability to bind Ptc and to induce AP expression (Table I). Modification of wild type Shh through the N-terminal cysteine with PEG 5000 maleimide also resulted in a large reduction in the activity of the protein in both assays (Table I). To control for possible effects of the maleimide chemistry on function, samples were also treated with N-ethylmaleimide (125 Da) and assayed for Ptc binding and the induction of AP on C3H10T1/2 cells (Table I). NEM treatment either had no effect or in most instances resulted in a slight increase in activity. Because NEM treatment was highly selective for the engineered cysteines and had no deleterious effect on activity, we selected the maleimide chemistry as the basis for all subsequent modification work.

When the activity data for the pegylated compounds were analyzed with respect to the position of the mutations in the crystal structure of Shh, the data revealed regions in the protein that were required for function. The engineered cysteines from mutants N69C, Y80C, and N91C for which pegylation had no effect on function all resided on the same surface of the protein from which the C terminus of the protein protrudes. Since a cholesterol is attached at or near the C terminus of Shh in its natural form (18, 19), this region is likely to be associated with the cell membrane and would not be expected to be involved in receptor binding. Since pegylation of all other mutants affected function, albeit to varying degrees, little additional information could be extracted from the data. In the crystal structures of mouse and human Shh, a Zn$^{2+}$ ion is coordinated to the protein in an arrangement similar to that seen in zinc hydrolases such as carboxypeptidase A. It was interesting that mutants S135C and S177C, which were designed to incorporate maleimide probes on either side of the cysteine containing the metal-binding site, retained partial activity. If Shh activity required the metal-binding site to interact directly with another protein, then PEG modification at those sites should have totally inactivated the protein. In recent studies, where mutagenesis was used to eliminate key amino acids that would be needed for enzymatic activity if the Zn$^{2+}$ had a catalytic role, the mutations were without effect, further indicating that catalysis is not required for hedgehog function (35, 36).

Refinement of the Structure-Activity Data Using Alternative Probes—Because the PEG 5000 modification divided the mutants into three groups (active, inactive, and partially active) and NEM alone had no clear effect on function, we reasoned that it would be possible to refine further the analysis by selecting and analyzing the effects of modifying groups of other sizes and shapes. Maleimide-containing probes are the most useful for this type of analysis because there are over 100 different commercially available N-substituted maleimide reagents of varying sizes and shapes. Four additional maleimide probes were selected, three smaller versions containing either a single aromatic ring (hydroxyphenyl), three aromatic rings (naphthyl), or four rings (pyrenyl), and a larger form containing a PEG 20,000. Results from these analyses are summarized in Table II. When the Shh mutants were modified with PEG 20,000, the same three mutants (N69C, Y80C, and N91C) that retained function with PEG 5000 remained active after derivatization with the PEG 20,000, whereas mutants S135C and G169C, which were partially inhibited by the PEG 5000, exhibited greater inhibition by modification with the PEG 20,000 PEG. The other mutants that were inactive after modification with PEG 5000 were also inactive after modification with 20,000 PEG.

| Shh variant | Unmodified | NEM | HPE | Naphth | Pyrene | PEG 5000 | PEG 20,000 |
|------------|------------|-----|-----|--------|--------|---------|------------|
| C241/A192C | 0.3        | 0.2 | 0.2 | 0.2    | 0.2    | 0.3     | 0.3        |
| +N50C      | 0.4        | 0.3 | 0.5 | 1.5    | 3      | 4       | 8          |
| +N69C      | 0.4        | 0.2 | 0.4 | 0.4    | 0.5    | 0.3     | 0.3        |
| +Y80C      | 0.2        | 0.2 | 0.2 | 0.2    | 0.3    | 0.2     | 0.3        |
| +N91C      | 0.5        | 0.2 | 0.2 | 0.3    | 0.3    | 0.3     | 0.2        |
| +N115C     | 1.5        | 2   | 2   | 4      | 3      | <20     | >20        |
| +S135C     | 0.4        | 0.2 | 0.2 | 0.3    | 0.3    | 0.6     | 2          |
| +S156C     | 0.4        | 0.2 | 0.2 | 0.6    | 3      | 2       | 2          |
| +G169C     | 0.3        | 0.3 | 0.2 | 0.2    | 0.3    | 0.5     | 2          |
| +S177C     | 1          | 2   | 2   | 2      | 3      | 3        | 5          |

When the Shh mutants were treated with the smaller maleimide probes and tested for function, we observed a clear dependence of the size and shape of the modifying group on its ability to interfere with function. Of the six sites where modification with 5000 PEG impacted function (Cys-24, Asn-50, Asn-115, Ser-177, Ser-135, and Ser-156), only two, Asn-50 and Ser-156, were affected by the addition of the pyrenyl moiety. Pyrene maleimide treatment resulted in a 10-fold loss in the potency of the N50C mutant and a 15-fold loss for the S156C mutant as compared with the NEM-treated controls. The activity of mutant N50C was also reduced by 5-fold following modification with the naphthyl moiety and by 1.7-fold after addition of the hydroxyphenyl moiety, whereas the activity of mutant S156C was reduced by 3-fold by the naphthyl group and was not affected by the hydroxyphenyl moiety. Thus by use of this steric interference analysis, it was possible to distinguish between the mutants by the size of the modifying group needed to block function.

Mapping Shh-Ptc Interactions by Cross-linking—Shh-Ptc interactions were also evaluated using cross-linking to probe the proximity of the mutated sites to Ptc (Fig. 3). For these analyses, the Shh mutants were first modified with the photoreactive cross-linker benzophenone-4-maleimide. Ptc-transfected 293 cells were incubated with the Bm-activated Shh conjugates, exposed to UV light, and lysates from the cells subjected to Western blotting. The blots were probed first with an anti-Shh antibody and then with an anti-Ptc antibody. When the blots were probed for Ptc expression (Fig. 3A), three bands were observed with masses of approximately 80, 170, and 270 kDa. These bands were not detected in lysates from untransfected
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293 cells (data not shown). Based on the sequence of the ptc construct, we expected Ptc to have an apparent mass of 170 kDa, consistent with the prominent band seen at that position. Whereas the other forms have been reported previously and presumably are related to Ptc (22, 30), their structures are not known. When the same blot was probed with anti-Shh antibody (Fig. 3B), all of the mutants except the parent protein C24II/A192C, N89C, and Y80C formed cross-linked complexes with Ptc as evidenced by the presence or absence of the band at 170–180 kDa. This band was not detected in cell lysates from Ptc-transfected or non-transfected 293 cells that had not been subjected to cross-linking (data not shown). The fact that most of the mutants formed a cross-linked complex with Ptc indicates that most of the surface of Shh is in relatively close proximity with Ptc. We have not characterized the other cross-linked bands that were detected by the anti-Shh antibody.

Although the results from cross-linking are similar to the blocking data we observed with the PEG-modified mutants, we had expected that the data would more closely resemble the effect of pyrene modification on activity. Several possibilities could account for the data. First, loss of function is a more rigorous test for association than cross-linking, since cross-linking only requires that the Shh and Ptc be localized within the span of the cross-linker, i.e., approximately 20 Å, during the lifetime of the activated cross-linker, whereas loss of function requires that the interaction be close enough that the probe can interfere with binding. Second, many of the probes used in these studies can assume a planar orientation because of free rotation around the bond between the nitrogen on the maleimide and the functional group. In this orientation the groups would be less disruptive than the size indicates. In addition to our analysis of sites on Shh that are associated with Ptc, the benzophenone-modified mutants should serve as valuable probes for mapping sites on Ptc that are associated with Shh as well as for studying interactions between Shh and other receptors such as Ptc-2 and HIP.
supports the hypothesis that this region on Shh interacts with the cell membrane. All other sites are sensitive to pegylation. Of the six sites where pegylation affected function, only two (N50C and S156C) lost activity following modification with pyrene maleimide (Fig. 5C). These sites are located on surface loops on either side of where the N terminus protrudes from the surface of the protein. We have shown previously that the N-terminal cysteine is a critical element for function, and modifications at this site can result in up to a 30-fold increase in potency (17) or loss of function (30) when evaluated in the C3H10T1/2 assay. The published crystal structure of murine Shh (20) is of an N- and C-terminally truncated form of the protein containing residues 34–195, and the first residue visible in the electron density was lysine 39. Thus, no information on the N terminus could be elucidated from the murine structure. In the crystal structure of the human Shh C24II mutant, the entire N terminus is visible in the electron density (Fig. 1). A striking feature of the N-terminal region is its extended conformation; the N-terminal amino group is located approximately 30 Å away from the globular domain of Shh. This unusual conformation is stabilized by crystal contacts. Specifically, Phe-30 and Pro-26 make extensive hydrophobic contacts with an aromatic region containing residues Phe-47, Trp-172, Tyr-174, and His-182 near the metal binding cleft of a symmetry-related molecule.

Recently, Beachy and co-workers (35) published a study where they mapped Ptc-binding regions on Shh by conventional mutagenesis using evolutionarily conserved surface residues as targets. In their study, four mutants were generated that together contained 20 different mutations. Each mutant defined a geographic surface of Shh (referred to as SA, SB, SC, and SD). Of these, only the SC surface contributed significantly to Ptc binding. Three additional mutants were generated that each contained two of the six SC mutations. These additional mutants exhibited clear but reduced effects on Ptc binding and signaling. Here, using steric interference to map Ptc-Shh interactions, we find that the same overall region of Shh is involved in Ptc binding. Two of the sites that we mutagenized (Ser-156 and Ser-177) are on the SC surface, and modifications to both affected function. Ser-156 was particularly sensitive to modification as evident by the large reduction in potency following modification with pyrene maleimide. Modifications to the S177C mutant only affected function when the larger PEG attachments were used. In our analysis, modification of N50C with pyrene also interfered with Ptc-Shh interactions. Asn-50 is near Ser-156 in the Shh structure and lies on the interface between the SB and SC surfaces. Together with the data from Fuse et al. (35), our findings stress the importance of the region in Shh that is defined by Ser-156 and Asn-50 for Ptc binding. We also determined that N115C, which lies in the SA surface, and Gly-169 and Ser-135, which were outside of the regions tested, affected binding but only when modified with the larger PEG attachments. These findings, together with our data from cross-linking, suggest that Shh-Ptc interactions may cover a larger surface of Shh than was previously predicted.

In addition to mapping sites on Shh that interact with Ptc, we also used the cysteine mutants to characterize the binding epitope for mAb5E1, a neutralizing antibody that disrupts binding of Shh to Ptc. Whereas the 5E1 site was localized to within the SC surface by Fuse et al. (35), we determined that Ser-177 forms part of the binding epitope and thus further localized the binding site within this region. Our data reveal that 5E1 binding and Ptc binding are at distinct but close and potentially overlapping sites on Shh.
A significant advantage of the mapping analysis by steric interference over conventional mutagenesis studies is that the proximity of a specific group on a ligand with its receptor can be mapped even if the ligand residue and the receptor are not in direct contact. This is highlighted here by the fact all of the Shh cysteine substitution mutants tested were active prior to modification, but with each incremental increase in the size of the probe, modification blocked function in a larger percentage of the mutants. Since the pattern of inhibition seen with each probe is a subset of that seen with the next larger size probe, the changes in the patterns presumably reflect differences in the distance between the interacting sites. This notion is particularly apparent for the smaller probes that were tested. Although we successfully utilized the 5000 and 20,000 PEG maleimide agents to map Shh-Ptc interactions on a gross level, the linear structure and flexibility of the PEGs limit their utility for this type of analysis. One approach that would greatly enhance the method would be to rationally design a series of small globular probes of rigid structure that could be used to define more precisely actual distances between interacting sites.

Although the concept of mapping by steric interference stems from antibody strategies for epitope mapping where antibodies frequently block function through steric effects rather than by directly binding at active sites, this is the first study to our knowledge where targeted probes of varying sizes have been used in such a manner. Whereas conventional mutagenesis (35) and the steric interference strategy described here both allow for the mapping of functional epitopes within the Shh structure, loss of function by mutagenesis is often difficult or impossible to attain if a large surface of a protein is involved in binding, and in several instances, mutagenesis studies have produced erroneous conclusions because loss of function was unrelated to ligand/receptor binding (37). The steric interference method we describe should provide an alternative to these types of approaches.

In summary, we have used a novel method probing structure-function relationships for Shh-Ptc interactions where we mutagenized Shh and inserted cysteines at surface positions that could then be specifically derivatized with groups of different sizes and shapes. The effects of the modifications on function were then determined. Based on these analyses, key regions in the structure that were involved in Ptc binding and 5E1 binding were mapped that give a new view of how Shh interacts with Ptc. The mapping strategy should be readily applicable to any protein but is likely to prove particularly valuable where structural data exists.

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