Functional Characterization of Sonic Hedgehog Mutations Associated with Holoprosencephaly*

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Mutations of the developmental gene Sonic hedgehog (SHH) and alterations of SHH signaling have been associated with holoprosencephaly (HPE), a rare disorder characterized by a large spectrum of brain and craniofacial anomalies. Based on the crystal structure of mouse N-terminal and Drosophila C-terminal hedgehog proteins, we have developed three-dimensional models of the corresponding human proteins (SHH-N, SHH-C) that have allowed us to identify within these two domains crucial regions associated with HPE missense mutations. We have further characterized the functional consequences linked to 11 of these mutations. In transfected HEK293 cells, the production of the active SHH-N fragment was dramatically impaired for eight mutants (W117R, W117G, H140P, T150R, C183F, L271P, I354T, A383T). The supernatants from these cell cultures showed no significant SHH-signaling activity in a reporter cell-based assay. Two mutants (G31R, D222N) were associated with a lower production of SHH-N and signaling activity. Finally, one mutant harboring the A226T mutation displays an activity comparable with the wild-type protein. This work demonstrates that most of the HPE-associated SHH mutations analyzed have a deleterious effect on the availability of SHH-N and its biological activity. However, because of the lack of correlation between genotype and phenotype for SHH-associated mutations, our study suggests that other factors intervene in the development of the spectrum of HPE anomalies.

Holoprosencephaly (HPE) is the most common brain anomaly in humans, involving abnormal formation and septation of the developing central nervous system and occurring in 1 in 16,000 live births and 1 in 250 spontaneous abortion cases (1). The clinical manifestations of the disease are variable, ranging from a single cerebral ventricle and cyclopia to minor anomalies of midline structures. The etiology of HPE is extremely heterogeneous, including genetic factors and environmental agents. The majority of HPE cases are sporadic, although autosomal dominant familial cases have been described. Genetic studies have shown that more than 10 chromosomal loci are implicated in HPE and 7 genes have been already identified: Sonic hedgehog (SHH) isolated from the human critical region HPE3 on chromosome 7q36 (2, 3), ZIC2 (13q32; HPE5) (4), SIX3 (2p21; HPE2) (5), TGF (18p13.1; HPE4) (6), Patched (PTC) (9q22) (7), TDGF1 (3p21.31) (8), and GLI2 (2q14) (9).

SHH mutations including nonsense and missense mutations, but also deletions and insertions constitute about 50% of the known HPE mutations as reported by several genetic screenings (10–16). If the deleterious role of nonsense or frameshift mutations is evident in the pathogenesis of genetic diseases, the implication of missense mutations has to be proved. Moreover, the ability to discriminate between a loss-of-function mutation and a silent polymorphism is important for genetic testing of inherited diseases like HPE, where the opportunity for genetic counseling exists (17).

SHH is a morphogen molecule involved in embryonic development including the induction of the floor plate and the establishment of the ventral polarity within the central nervous system (18). SHH is synthesized as a precursor that undergoes autocatalytic cleavage into a highly conserved N-terminal domain (SHH-N) responsible for the signaling activities of the molecule and a more divergent C-terminal domain (SHH-C) implicated in the autoproteolysis reaction and the addition of a cholesterol moiety cotranslationally anchored to the C-terminus of SHH-N (19). This cholesterol molecule is presumably important for tethering the protein to the plasma membrane and, thus, restricts the tissue localization of hedgehog signaling. The addition of another lipid, a palmitoyl group, on the N-terminal cysteine residue of SHH-N dramatically increases the biological activity of the protein (20, 21). Thus, these modifications of SHH may exert a key role in targeting the protein to lipid rafts (22) that could represent functional platforms for SHH signaling. Diffusion of a soluble cholesterol-modified multimeric form of mouse Shh-N has been proposed to mediate long range signaling during embryonic development (23).

SHH mediates its action via a receptor complex associating two transmembrane proteins; that is, PTC, displaying a transporter-like structure, and Smoothened, presumably belonging to the G protein-coupled receptor superfamily. The repression exerted by PTC on Smoothened is relieved when SHH binds PTC, which leads to a complex signaling cascade involving the transcription factors of the Gli family and to the activation of target genes including PTC itself (18, 24). Two PTC genes have been molecularly cloned (25–27). During embryonic develop-

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** The abbreviations used are: HPE, holoprosencephaly; AP, alkaline phosphatase; PTC, Patched; SHH, Sonic hedgehog; SHH-N, Sonic hedgehog N-terminal protein; SHH-C, Sonic hedgehog C-terminal protein; WT, wild-type.
SHH Mutants Implicated in HPE

EXPERIMENTAL PROCEDURES

Modeling the Human SHH-N and SHH-C Proteins—The three-dimensional models of the human SHH-N and SHH-C domains were constructed from the x-ray structures of mouse Shh-N (35) and Drosophila hh-C (36), respectively. After alignment of the amino acid sequences of the above-cited proteins using standard parameters of the ClustalW program (37), human SHH-N and SHH-C proteins were modeled using the BIOPOLYMER module of the SYBYL 6.9 package (TRIPOS Assoc., Inc., St. Louis, MO). Two insertions of one and three residues in SHH-C were modeled using a classical loop search procedure as already described (38), whereas a 19-residue insertion could not be modeled. Standard geometries for the mutated side chains were given by the BIOPOLYMER module of SYBYL. Whenever possible, the side-chain torsional angles were kept to the values occurring in the x-ray templates. Otherwise, a short scanning of side chain angles was performed to remove steric clashes between the mutated side chain and the other amino acids. After the heavy atoms were modeled, all hydrogen atoms were added, and the protein coordinates were then minimized with AMBER (39) using the AMBER95 force field (40). The minimizations were carried out by 1000 steps of steepest descent followed by conjugate gradient minimization until the root-mean-square gradient of the potential energy was less than 0.05 kcal/mol/A. A twin cut-off (10.0, 15.0 Å) was used to calculate non-bonded electrostatic interactions at every minimization step, and the non-bonded pair-list was updated every 25 steps. A distance-dependent (ε = 4) dielectric function was used.

Site-directed Mutagenesis—To mutate SHH amino acids, the full-length cDNA encoding the human wild-type (WT) SHH (GenBank accession L93518), kindly provided by Dr. O. Tahini (Harvard Medical School, Boston, MA), was used. Mutations G31R, W117G, and I338T (41), kindly provided by Dr. O. Tahini, were made using the QuickChange XL site-directed mutagenesis kit (Stratagene) according to the manufacturer’s instructions with primers that are available upon request. The mutations were confirmed by automated DNA sequence analysis using the BigDye® Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, CA) and the ABI Prism 3100 genetic analyzer. The WT and mutated SHH cDNAs were subcloned into the pRK5 vector for use in transfection experiments.

Cell Culture—HEK293 and C3H10T1/2 cells (ATCC) were cultured at 37 °C in Dulbecco’s modified Eagle’s medium (Invitrogen) supplemented with 10% heat-inactivated fetal calf serum (Eurobio, Les Ulis, France). Metabolism of SHH Mutants Implicated in HPE

Animal models support the concept that mutations resulting in modulation of SHH activity-signaling pathway can cause HPE. The phenotype observed in Shh knockout mice (31) associating cyclopia and loss of normal ventral specification in the forebrain mimics clinical manifestations of HPE. A percentage of mice with increased PTC activity driven by the nestin enhancer display a fusion of the lateral ventricles consistent with HPE (32). Smoothed homozygous knockout mice also present cyclopia and HPE (33). Finally, homozygous null mutant for Gli2 displays a single central maxillary incisor constituting a microform of HPE (34).

We have now developed a three-dimensional model of human SHH-N and SHH-C domains that were used to highlight important regions associated with HPE mutations within both peptides. We have mutated a panel of amino acid residues associated with HPE and distributed both in SHH-N and SHH-C. We present biochemical and functional analysis of the mutated proteins and discuss their functionality in the development of HPE.
one containing the highest number of SHH-N-mutated residues, includes amino acids located in two α-helices (Q100H, A110D, I111F, N115K, W117R, W117G, T150R) and in the C-terminal end of SHH-N (E188Q). These residues lie at the surface of the protein, suggesting that they may be implicated in signaling and potentially in protein interactions. The second region involves residues implicated in or surrounding the Zn²⁺ binding site. These mutations would modify residues directly implicated in Zn²⁺ coordination such as His-140 or affect such residues such as Cys-183, which is adjacent to His-182. The G31R mutation affects a residue located in the N-terminal region that has been shown to be away from the globular domain of Shh-N and to possibly make hydrophobic contacts with His-182 of a symmetrical SHH-N molecule (43).

In the SHH-C model we observed that Asp-243, Thr-267, and His-270 residues are facing the Cys-198 residue, in agreement with their implication in the internal thioester re-arrangement (Fig. 3B) as previously shown in Drosophila hh (36). A first cluster of SHH-C residues mutated in HPE comprises Asp-222, Val-224, and Ser-236 and are located in the same region of the protein (Fig. 3B). Except Asp-222, the two other amino acids are not readily accessible for protein interactions since they appear to be buried in the SHH-C ternary structure. A second cluster of residues including Pro-347 and Ile-354 is found in the C-terminal region, which is incompletely shown in our model (Fig. 3B). This region has been proposed to bind cholesterol and to be responsible for its transfer during the autoprocessing reaction. Arg-381, Ala-383, Pro-424, and Ser-436 (not shown in our model) probably belong to this cluster. The other mutations do not segregate in a particular region (Fig. 3B). However, the L271P mutation, which is located next to His-270, proposed to intervene in the thioester formation (see above) probably introduces a dramatic change in the folding of the SHH-C protein, further blocking the amino acid re-arrangement. We were not able to get more insights from the model either for the mutation affecting the Val-332 residue that lies at the surface of SHH-C or for the Gly-290 residue located in the 19-residue insertion region.

Analysis of Human WT SHH Processing in Transfected HEK293 Cells—To examine the effect of mutations on the processing of SHH and on its biological activity, we first characterized the expression of WT SHH transiently transfected in HEK293 cells. Synthesis and secretion of the protein were further monitored by Western blot analysis using two polyclonal antisera recognizing specifically SHH-N (167Ab) (42) or SHH-C (1229Ab) (Fig. 1). Both sera detected SHH precursor protein as 48–51-kDa polypeptides in homogenates from transfected cells, in agreement with the predicted molecular mass deduced from SHH sequence (44). These polypeptides might reflect SHH protein before and after signal peptide cleavage as previously observed for chicken and mouse Shh proteins (45). As expected, SHH-N and SHH-C fragments were further identified as a major (Fig. 4A) and a faint (Fig. 4B) polypeptide migrating with a relative molecular mass of 22 or 33 kDa,
respectively, indicating that the autoprocessing reaction had occurred.

SHH-N was further released in the culture medium of these transfected cells as a 22-kDa polypeptide (Fig. 4A). A broad signal migrating with a relative molecular mass of 35–38 kDa was also detected in the culture medium, indicating that SHH-C fragment was also secreted (Fig. 4B). The decrease in SHH-C mobility observed in the culture medium is consistent with glycosylation maturation of N-linked carbohydrates as previously reported for Drosophila hh, chicken, and mouse Shh (45, 46). All these signals were absent in homogenates and media from mock cell preparations, thereby indicating their specificity (Fig. 4).

**Biochemical and Functional Analysis of Missense Mutations Located within the SHH-N Domain**—We decided to model by site-directed mutagenesis 11 of these missense mutations identified in our model. These mutants were transiently transfected in HEK293 cells, and their expression was analyzed by Western blot. The expression pattern of the first mutant examined harboring the G31R mutation was comparable with that of WT SHH, except for the appearance of a band above the major 22-kDa SHH-N detected with WT sample. However, SHH-N-secreted peptide was identified as a major 21-kDa peptide instead of 22 kDa for the WT protein (Figs. 5A and 6C). We further compared the in vitro biological activity of the WT and G31R mutant SHH by measuring their ability to induce AP activity in C3H10T1/2 cells. This assay represents a reliable measure of the differentiation of these cells to an osteoblast lineage (47). The culture media from HEK293 cells transfected with either the WT- or G31R-SHH were applied to osteoblast lineage (47). The culture media from HEK293 cells transfected with either the WT- or G31R-SHH were applied to osteoblast lineage (47).

**TABLE I**

Comparison of genetic and functional traits linked to SHH mutations associated with HPE

For cDNA numbering (c.), +1 corresponds to the A of the ATG translation initiation codon, and for protein numbering (p.), +1 corresponds to the initiator codon. The GenBank access number for SHH cDNA is NM_000193.2. A, alobar HPE; SL, semilobar HPE; L, lobar HPE; Min, minor signs of HPE spectrum; AF, atypic features of HPE; ND, not defined in the reference article; *, presence; †, absence; *+, presence of a mutation in another HPE gene (c.G696A, mutation in ZIC2; c.1132delC, mutation in TGIF). References for clinical data are listed in the last column.

| Mutation (+1 is the A of the ATG initiator) | Amino acid change | Exon | HPE type | Mutation in kindred | Clinical signs in kindred | AP activity (present study)* | SHH-N peptide (present study)* | Reference |
|-------------------------------------------|------------------|------|----------|---------------------|--------------------------|-----------------------------|-------------------------------|-----------|
| c.9_10insGCTGT | Frameshift | 1 | A | ND | ND | ND | 11 |
| c.G17C | p.R67T | 1 | SL | ND | ND | ND | 16 |
| c.38_45del | Frameshift | 1 | L | ND | ND | ND | 11 |
| c.T50C | p.L17P | 1 | L | ND | ND | ND | 59 |
| c.C72A | p.C24X | 1 | A | ND | ND | ND | 16 |
| c.G91A | p.G31R | 1 | ND | + | + | 1+ | 1+ | 3 |
| c.211delG | Frameshift | 1 | SL | + | + | ND | 16 |
| c.A263T | p.D88V | 1 | A | + | - | ND | 11 |
| c.C298T | p.Q100X | 1 | ND | + | + | ND | 3 |
| c.G300C | p.Q100H | 1 | SL | – | ND | ND | 12 |
| c.A307T | p.K105X | 2 | ND | + | – | ND | 3 |
| c.T316_321del | Frameshift | 2 | A | – | ND | ND | 16 |
| c.C329A | p.A110D | 2 | Min | + | –/+ | ND | 16 |
| c.A331T | p.I111F | 2 | Min | + | /– | ND | 13 |
| c.G345A | p.N115K | 2 | L | + | – | ND | 11 |
| c.T349G | p.W117G | 2 | ND | + | + | 0 | 3 |
| c.T349G | p.W117R | 2 | ND | + | + | 0 | 3 |
| c.G383A | p.W129X | 2 | A | + | + | ND | 15 |
| c.G388T | p.E130X | 2 | SL | + | ND | ND | 16 |
| c.A419C | p.H140P | 2 | ND | + | –/+ | ND | 14 |
| c.C449G | p.T150R | 2 | SL | – | ND | ND | 16 |
| c.G475G | p.Y158X | 2 | AF | + | – | ND | 12 |
| c.527_535del | Frameshift | 2 | A | + | ND | ND | 16 |
| c.G548T | p.E188Q | 2 | SL | + | – | ND | 14 |
| c.C562T | p.Q209X | 3 | SL | + | – | ND | 11 |
| c.G664A | p.D222N | 3 | SL | + | –/+ | ND | 11 |
| c.G664A | p.D280N | 3 | SL | + | /– | ND | 11 |
| c.T671A | p.V224E | 3 | SL | + | – | ND | 16 |
| c.G676A | p.A383T | 3 | ND | + | – | ND | 16 |
| c.G676A | p.A383T | 3 | ND | + | – | ND | 16 |
| c.T706C | p.I354T | 3 | L | ND | ND | ND | 10, 11 |
| c.788_806del | Frameshift | 3 | L | ND | ND | ND | 11 |
| c.T812C | p.L271P | 3 | AF | + | + | ND | 11 |
| c.G850T | p.E284X | 3 | AF | + | + | ND | 11 |
| c.G888A | p.G290D | 3 | SL | + | – | ND | 11 |
| c.C895C | p.V331A | 3 | Min | + | – | ND | 16 |
| c.C1040A | p.P347Q | 3 | A | + | + | ND | 16 |
| c.T1061C | p.I354T | 3 | A | + | ND | ND | 16 |
| c.1132_1140del | Frameshift | 3 | SL | + | – | ND | 11 |
| c.G1142C | p.R381P | 3 | SL | + | ND | ND | 16 |
| c.G1147A | p.A383T | 3 | ND | ND | ND | 0 | 10 |
| c.1210_1224del | Frameshift | 3 | ND | – | ND | ND | 11 |
| c.C1270G | p.C424A | 3 | SL | + | – | ND | 11 |
| c.C1307T | p.S436L | 3 | SL | ND | ND | ND | 11 |

*Values for AP activity and for intensity of the soluble 22-kDa SHH-N peptide are from the present study. Intensities: 0, not detectable; 1+, low; 2+, moderate; 3+, strong; 4+, comparable to WT.
G31R-mutated SHH-N peptides was dramatically reduced at the end of the experiment, indicating that this G31R mutant is highly susceptible to proteolytic degradation (Fig. 7, B–C).

The signals corresponding to SHH-N peptides were absent from homogenates or culture media of cells expressing SHH harboring the W117R, W117G, H140P, T150R, or C183F mutations, indicating a profound modification of the SHH-N expression pattern and, to a lesser extent, that of the SHH precursor (Figs. 5A and 6A and B). Indeed, the signal corresponding to the precursor protein (48–51 kDa) was greatly diminished for the T150R and C183F mutants (Fig. 6A). A faint signal corresponding to SHH-C peptide of W117R, W117G, and H140P mutants was detected in the culture medium of transfected cells (Fig. 5B) and was more evident after a longer exposure time of the film (data not shown). This observation suggests that at least a limited autoprocessing reaction of the mature protein had occurred in these three mutants. In agreement with the absence of detectable released SHH-N peptides (Figs. 5A and 6C), we did not detect a significant functional activity in the culture medium of HEK293 cells transfected with the five mutants in the differentiation assay (Fig. 7A). These results suggest either a susceptibility to proteolysis of W117R, W117G, H140P, T150R, and C183F mutants or an impairment of their autoproteolysis reaction or both.

Biochemical and Functional Analysis of Missense Mutations Located within the SHH-C Domain—The D222N and A226T mutations affect residues located in the Hint (Hedgehog Intein) module, which is related to self-splicing proteins (36). Asp-222
and to a lesser extent Ala-226 residues are highly conserved between species (Fig. 2B) (36), but a role in autoprocessing of SHH protein has not yet been described. We observed that the precursor protein of mutants harboring the D222N and A226T mutations significantly increased compared with the WT (n = 3, p < 0.05; Figs. 5A and 6A), suggesting an impairment of the autoprocessing reaction. In agreement with this hypothesis, membrane-associated SHH-N as well as soluble SHH-N and SHH-C peptides from the D222N mutant were reduced significantly compared with the WT protein (n = 3, p < 0.05) (Figs. 5, A and B, and 6, B and C), and the corresponding functional activity represented 53 ± 5% (mean ± S.E., n = 7, p < 0.005) of WT activity (Fig. 7A). However, the A226T mutant did not show any further differences compared with the WT.

For cells transfected with the mutant harboring the L271P mutation, Western blot analysis using the anti SHH-N serum revealed the presence of several peptides that might correspond to proteolytic fragments of the precursor protein in both the cell homogenate and culture medium (Fig. 5A). Interestingly, the anti SHH-C serum did not detect the fragments corresponding to either the mature protein or the processed peptides (Fig. 5B), suggesting again that proteolysis had occurred. Alternatively, the mutation may have modified the three-dimensional structure of the protein, preventing recognition by SHH-C antiserum. The deleterious effect of the L271P mutation was confirmed by the very low AP activity (Fig. 7A).

The two last mutations studied belong to the cluster comprising residues mutated in the carboxyl end of SHH-C. The peptidase to a lesser extent Ala-226 residues are highly conserved between species (Fig. 2B) (36), but a role in autoprocessing of SHH protein has not yet been described. We observed that the precursor protein of mutants harboring the D222N and A226T mutations significantly increased compared with the WT (n = 3, p < 0.05; Figs. 5A and 6A), suggesting an impairment of the autoprocessing reaction. In agreement with this hypothesis, membrane-associated SHH-N as well as soluble SHH-N and SHH-C peptides from the D222N mutant were reduced significantly compared with the WT protein (n = 3, p < 0.05) (Figs. 5, A and B, and 6, B and C), and the corresponding functional activity represented 53 ± 5% (mean ± S.E., n = 7, p < 0.005) of WT activity (Fig. 7A). However, the A226T mutant did not show any further differences compared with the WT.

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SHH-N peptides corresponding to either I354T or A383T mutants were not detected in the cell homogenates, suggesting that the autoprocessing reaction had not occurred in these proteins. The A383T mutation led to the accumulation of the precursor protein before its secretion in the culture medium compared with the WT (n/H11005 3, p/H11021 0.05) (Figs. 5 and 6A). However, we did not detect a significant increase of AP activity when the culture medium was assayed on C3H10T1/2 cells, showing that the secreted precursor protein observed in the culture medium was not active in this assay (Fig. 7A).

**DISCUSSION**

We have modeled for the first time a large panel of SHH mutations associated with HPE. After transfection of the mutant proteins in HEK293 cells, we have analyzed their biological activities by using the hedgehog-induced response of the

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**Fig. 6. Quantification of SHH precursors and SHH-N polypeptides from HPE mutants.** Densitometry analysis of autoradiography from Western blots analyzed with anti SHH-N serum was performed as described under “Experimental Procedures.” Data are means ± S.E. from at least three independent experiments. Relative intensity of the signals corresponding to the 48–51-kDa SHH precursor bands in cellular homogenates (% of WT; smaller size for L271P and A383T) (A), the 22-kDa SHH-N polypeptide in cellular homogenates (% of WT) (B), and the 21-kDa (white bar) and 22-kDa (gray bar) polypeptides in culture media (% of WT 22-kDa signal) (C). *, p < 0.05 compared with WT.

**Fig. 7. Functional analysis of SHH alleles in C3H10T1/2 cells reporter assay and susceptibility to proteolytic degradation of the SHH-N fragments.** A, the relative potencies of WT and mutated SHH were assessed in C3H10T1/2 cells measuring the AP activity induced by conditioned media derived from transfected HEK293 cells. Basal (mock) and WT SHH-induced AP activity correspond to 0.19 ± 0.01 and 1.89 ± 0.27 optical density units, respectively. The data are expressed as % of the WT-induced AP activity and are the mean ± S.E. from 3–7 independent experiments performed in quadruplicate. *, p < 0.005 compared with WT. B, samples (15 μl) of conditioned media derived from HEK293 cells transiently transfected with WT or SHH mutants were harvested at the start (J = 0) and at the end (J = 5) of the AP reporter assay and analyzed by Western blotting using the anti SHH-N antibody. Molecular mass markers (kDa) are indicated on the right. C, densitometry analysis of the signals corresponding to 21–22-kDa SHH-N peptides at J = 0 and J = 5 shown in B. The profile of the A226T mutant protein was comparable with that of the WT protein. The G31R signal was dramatically reduced at J = 5, indicating proteolysis.
C3H10T1/2 cells. Our results show that at least three different classes of inactivating SHH mutants exist. The first one includes mutants affecting the stability of the precursor protein or of SHH-N and displaying mutations located either within SHH-N (G31R, W117R, W117G, H140P, T150R, C183F) or SHH-C (L271P). The absence or low functional activity in the cell-based assay with these mutants is in agreement with the absence or the very low expression level of mutated SHH-N fragment in the supernatant of the transfected cells. The crystal structure of murine Shh-N has revealed the presence of a Zn$^{2+}$ ion coordinated by His-141, Asp-148, and His-183 that, respectively, correspond to His-140, Asp-147, and His-182 in human SHH-N. This arrangement is similar to that found in several Zn$^{2+}$ hydrolases and has led to the hypothesis that SHH might display hydrolase activity (48, 49). Denaturation studies conducted on the H140A and D147A SHH-N mutants have previously shown that the loss of Zn$^{2+}$ binding is accompanied by a misfolding of the proteins, which were, thus, highly susceptible to proteolytic degradation and very difficult to purify (49). One can speculate that the substitution of a histidine by a proline residue (H140P) would also impair Zn$^{2+}$ binding. In the same way the substitution of threonine (Thr-150) and cysteine (Cys-183) located next to the important Asp-147 and His-182 residues by an arginine and a phenylalanine, respectively, would also affect Zn$^{2+}$ binding. Thus, the H140P, T150R, and C183F SHH-N mutants might be very unstable and susceptible to proteolysis, which is in agreement with our results. This may be also the case for the W117R and W117G mutants. When expressed in vivo in the chick ventral neural tube or the forebrain, the W117R and W117G SHH mutants failed to induce appropriate transcription factors associated with SHH signaling (50). This study did not allow the conclusion that this was due to a reduced activity or another alteration of the mutant protein. Our present studies conducted with the W117R and W117G mutants indicate that the lack of activity would be primarily due to the lack of secreted SHH-N.

The G31R mutation is located in the N-terminal region of SHH-N and lies next to a phenylalanine (Phe-30) and a proline (Pro-26) postulated to make hydrophobic interactions with residues delimitating the Zn$^{2+}$ binding cleft of a SHH-N symmetrical molecule (43). Therefore, the G31R mutation might destabilize these interactions, leading to a protein with an increased susceptibility to proteolysis.

Our model of SHH-N indicates that several of these mutated residues belong to a cluster of amino acids that lie at the surface of the protein (Fig. 3A). Therefore, it is plausible that this region may interact directly with an as yet unidentified protein to stabilize or to prevent further degradation of SHH-N or, alternatively, of SHH protein before autoprocessing.

The Leu-271 residue is a highly conserved residue found in most inteins (36) residing next to His-270 and Thr-267. These two conserved residues have been demonstrated to participate directly in an internal thioester formation with Cys-258 in Drosophila hh-C (36), allowing further cholesterol transfer. The replacement of a leucine by a proline at position 271 would imply a profound modification of the ternary structure of the protein, not only preventing the thioester formation but also increasing its susceptibility to proteolysis as observed in our study.

The second class of mutations includes those that affect the autoprocessing reaction. The first one, D222N, is located in the N-terminal tail of SHH-C and found in almost all inteins (36). The conservative mutation replacing an aspartate by a slightly less charged asparagine might impair ionic interactions and modify the tertiary structure of the protein, possibly reducing the overall autoprocessing reaction leading to a reduced production of SHH-N.

The two other mutations, I354T and A383T, are located in the C-terminal tail of SHH-C and were characterized by a very low level of secreted SHH-N in the culture medium of transfected cells. The crystal structure and molecular studies of the autoprocessing domain of Drosophila hh have revealed the role exerted by the carboxyl domain. A construct lacking the last 63 amino acids at the C-terminal was able to form the thioester intermediate but failed to transfer cholesterol, and a direct role in cholesterol binding for this region has been proposed (36). Therefore, it is plausible that structural modifications introduced by the I354T and A383T, located in the same region, prevent further the binding and/or the transfer of the cholesterol molecule.

A226T belongs to the last class of mutations that do not change the biological activity of the mutated protein compared with the WT SHH. Whereas our data suggest an impairment of the autoprocessing reaction of the mutated protein, as indicated by the accumulation of the precursor protein, further in vitro and in vivo experiments are required for identifying functional differences between the WT and the mutated protein.

Interestingly, among 44 mutations reported in Table I, 26 are also found in the kindred. Nevertheless, the correlation between genotype and phenotype is elusive, as in 10 cases the SHH mutation in the kindred is not associated with evident signs of the disease. There is also a lack of correlation between the phenotype severity on the one hand and the alteration of the biochemical properties and biological activity of the associated mutant proteins on the other (Table I). This phenomenon may be due to penetrance differences or to the existence of modifier genes. HPE was first described as a “single gene” autosomal dominant disease with a proved genetic origin associated with haplo-insufficiency of SHH (51). Then, the description of patients presenting with mutations in both SHH and a second HPE gene (11) suggested that HPE was a multigenic disease (52).

Animal models of HPE confirm this hypothesis, as Shh$^{-/-}$ mice have features of HPE or die during embryonic development, whereas the heterozygous mice appear normal (31). Other animal models heterozygous for mutations in genes belonging to the Shh or TGFβ pathways show that only double mutants show signs of HPE (53, 54), suggesting a bigenic inheritance in mouse models. Several human genetic diseases such as Usher syndrome, insulin resistance, or Hirschsprung’s disease, are now described as multigenic diseases (55–57). Recently Gabriel et al. (58) conducted a genome scan in families with Hirschsprung’s disease and identified susceptibility loci at 3p21, 10q11 (receptor-tyrosine kinase for glial cell line-derived neurotrophic factor), and 19q12 necessary and sufficient to explain the recurrence risk and population incidence. They propose a multiplicative effect of these three loci where the 3p21 and 19q12 loci could be receptor-tyrosine kinase-dependent modifiers. This approach could serve as a model for dissecting a complex disease such as HPE, provided a sufficient number of families is available. Besides the effect of modifier genes, environmental or metabolic factors may contribute to variable expressiveness and explain the absence of a strict genotype-phenotype correlation.

In summary, we have to our knowledge characterized for the first time the biochemical and biological properties of a large panel of SHH mutations associated with HPE. We have shown that most of these mutations have a deleterious effect on the availability of the SHH-N fragment and its biological activity, evaluated by the AP reporter cell-based assay, which appears to be an appropriate functional assay, due to the correlation found between functional and biochemical data. However, because of the lack of correlation between genotype and pheno-
type for SHH-associated mutations, our study underlines the necessity for further careful analysis to understand the complex molecular and biochemical traits linked to HPE.

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