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A Sonic Hedgehog Missense Mutation Associated with Holoprosencephaly Causes Defective Binding to GAS1

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Holoprosencephaly (HPE) is a common birth defect predominantly affecting the forebrain and face and has been linked to mutations in the sonic hedgehog (*SHH*) gene. HPE is genetically heterogeneous, and clinical presentation represents a spectrum of phenotypes. We have previously shown that *Gas1* encodes a cell-autonomous Hedgehog signaling enhancer. Combining cell surface binding, *in vitro* activity, and explant culture assays, we provide evidence that SHH contains a previously unknown unique binding surface for its interaction with GAS1 and that this surface is also important for maximal signaling activity. Within this surface, the Asn-115 residue of human SHH has been documented to associate with HPE when mutated to lysine (N115K). We provide evidence that HPE associated with this mutation can be mechanistically explained by a severely reduced binding of SHH to GAS1, and we predict a similar result if a mutation were to occur at Tyr-80. Our data should encourage future searches for mutations in *GAS1* as possible modifiers contributing to the wide spectrum of HPE.

Holoprosencephaly (HPE)[2] is a developmental defect of the brain and face estimated to affect 1 in 250 conceptuses (1). Clinical presentation represents a spectrum of phenotypes, ranging from the most severe (alobar), where embryos have cyclopia and the prosencephalon fails to divide into hemispheres, to relatively mild defects (microform HPE) such as maxillary central incisor fusion, midfacial hypoplasia and clefting, and the presence of a single nostril (2). The use of mice as a model has proven invaluable for investigating the molecular and genetic causes of HPE. We have previously reported that microform HPE develops in growth arrest-specific gene 1 (*Gas1*) mutant mice (3, 4). Additionally, we determined that the 37-kDa, cell surface-presented, and glycosylphosphatidylinositol-anchored GAS1 protein binds to the secreted cell-cell signaling protein Sonic hedgehog (SHH) and that it functions as a cell-autonomous enhancer of SHH signaling activity (3, 5, 6). Consistently, the *Gas1* mutant phenotype is more severe when an allele of *Shh* is removed, supporting a genetic interaction between the two genes (3, 4). Given the strong evidence that mutations in *Shh* can cause HPE in mice and humans (7–11), we investigated the hypothesis that some of these mutations cause defective SHH signaling due to a failed interaction with GAS1.

Here we identify specific residues on SHH that are required for maximal binding to GAS1 and show, in both cell culture and explant culture assays, that these mutant SHH proteins have decreased signaling activity due to their defective interaction with GAS1. Significantly, one of these mutations has been associated with autosomal dominant HPE in a human family (9). These results lead us to propose that human embryos carrying this mutation may develop HPE due to a failed GAS1-SHH protein interaction.

EXPERIMENTAL PROCEDURES

Cell Culture Assays—All cDNAs used were of murine origin. The N-terminal fragment of SHH (SHH-N) A–D surface mutants in bacterial expression plasmids (12) were PCR-amplified and fused to AP in pcDNA3. WT SHH-N-AP conditioned medium (CM) and its mutant variants used here were produced as described (3). SHH-N-AP concentration of each CM was normalized according to AP activity.

Surface binding assays in COS7 cells were performed as described (3) with the following changes: 1) 1 μg of expression plasmid for PTC1 (C terminus deleted), GAS1, CDO, or HIP1 was transfected per well of a 12-well plate; 2) binding was at 4 °C for 50 min to reach equilibrium (not shown); 3) the Phospha-Light system (Applied Biosystems) was used to detect AP activity with 150 μl of each component used per well; and 4) chemiluminescence was detected with a SpectraMax M5 machine (Molecular Devices), nine points per well, 0.1 s of integration time. Activity assays in NIH3T3 cells were performed as described (3), except that a slightly higher concentration of SHH-N was needed; presumably, the AP tag causes a small decrease in signaling activity.

Mouse Limb Bud Explant Assay—The assay was adapted from Refs. 13 and 14. *Gas1*$^{+/-}$ mice (3) were interbred to obtain E10.5 WT and mutant embryos. Anterior halves of size-matched forelimb buds were dissected and cultured for 24 h in a 4-well dish (Nunc) precoated with 300 μl of 1% UltraPure L.M.P. Agarose (Invitrogen) made in BGJb medium (Invitrogen). For culturing, each well contained: 50 μl of SHH-N-AP CM, 10 μl of penicillin/streptomycin, 100 μl of fetal bovine serum, 2 μl of ascorbic acid (150 μg/ml final), and 538 μl of BGJb medium. A titration of SHH-N-AP was performed analogously to that as in Fig. 2*A*, and the dilution 1:20 (40 nM) was found to be just below saturation (not shown). Total RNA was isolated from explant using TRIzol reagent (Invitrogen). qRT-PCR for *Gli1* (15) was performed using M-MuLV reverse transcriptase (New England BioLabs), SYBR green (Molecular Probes), and a DNA Engine Opticon continuous fluorescence detector system (MJ Research). The -fold increase in *Gli1* levels was determined

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[2] The abbreviations used are: HPE, holoprosencephaly; SHH, Sonic hedgehog; SHH-N, N-terminal fragment of SHH; AP, alkaline phosphatase; WT, wild type; CM, conditioned medium; qRT, quantitative reverse transcription; E, embryonic day.

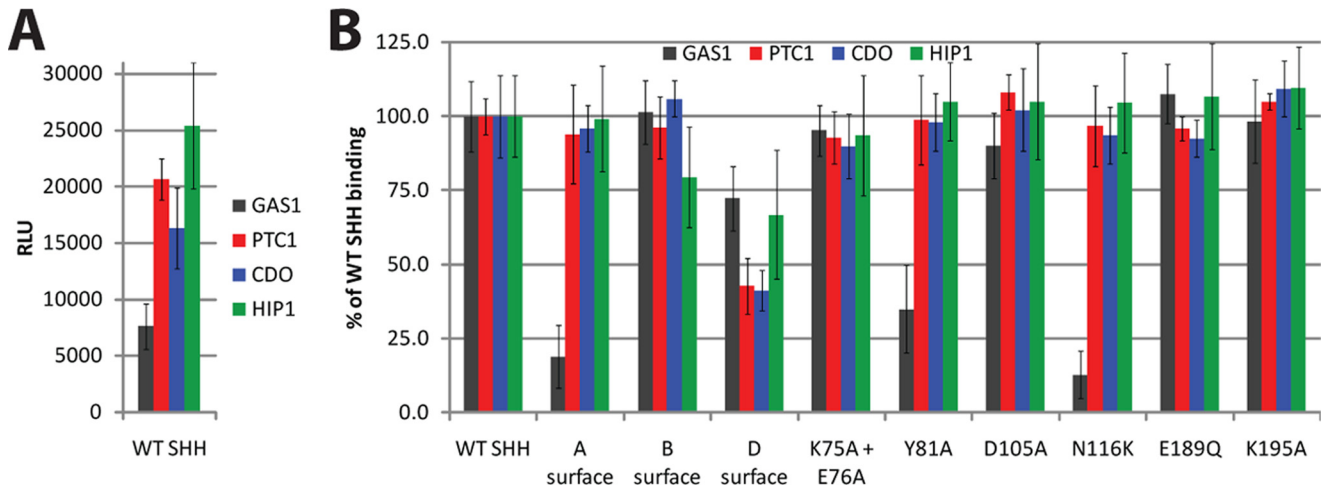

FIGURE 1. **SHH-N-AP cell surface binding to COS7 cells expressing each binding partner.** *A*, AP activity was measured in relative luminescence units (*RLU*) for WT SHH-N-AP binding. Values shown are after subtraction of binding to mock-transfected cells. Less binding to GAS1 is likely due to its higher K_d. Total surface protein levels were not controlled for as they were not relevant to this experiment. *B*, the binding of each SHH mutant is displayed as a fraction of WT SHH binding for each respective protein. All assays were performed in triplicate; *error bars* = 1 standard deviation.

using the following formula: -fold increase = $(2^{n\text{-control}})/(2^{n\text{-SHH}})$ where "*n*-control" and "*n*-SHH" are the mean threshold cycles of PCR done in triplicate on mock-treated and SHH-treated explants. qRT-PCR was performed for *Gapdh* for normalization (16).

RESULTS AND DISCUSSION

The secreted 19-kDa SHH-N is responsible for all of its known signaling activity. To further our understanding of the GAS1-SHH interaction, we tested various SHH-N mutants for their ability to bind to GAS1 in a cell surface binding assay where an alkaline phosphatase enzyme is tagged to the C terminus of SHH-N (SHH-N-AP) (5). Unlike bacterially produced SHH-N, SHH-N-AP produced in mammalian cells maintained the N-terminal palmitoyl modification important for maximum signaling activity (18). We first tested a series of SHH surface mutants where the conserved and surface-presented residues were grouped onto four distinct faces of the protein and mutated to alanine (SHH mutants A–D) (12). We compared their GAS1 binding ability with three other SHH binding proteins: Patched1 (PTC1), the apparent receptor for SHH; CDO, a cell surface signaling enhancer (with no homology to GAS1); and Hedgehog-interacting protein 1 (HIP1), a cell surface antagonist of SHH (12, 19–21). A concentration of 20 nM for each SHH-N-AP was used for binding, which was below the binding saturation for each of the tested binding partners (not shown). We found that the SHH A mutant had greatly decreased binding to GAS1, whereas it bound normally to PTC1, CDO, and HIP1 (Fig. 1). The B surface mutant bound normally to GAS1, PTC1, and CDO but slightly less to HIP1. The D mutant partially disrupts binding to all. The C mutant did not make a functional protein in mammalian cells, possibly due to misfolding. The PTC1 and CDO binding results for SHH A, B, and D are consistent with previous studies (12, 22). These results indicate that residues on the A surface are uniquely required for the GAS1-SHH interaction.

We noted that 2 of the residues changed in the A mutant (Asn-116 and Glu-189) have corresponding mutations in human SHH that have been implicated in HPE: N115K and E188Q (9, 10). To determine the GAS1 binding contribution of these residues, we introduced them as single residue mutants in the SHH-N-AP. The 5 other changed residues on the A mutant do not currently have reported human-associated mutations, so we created 4 new mutants changing each of these residues to alanine (K75A/E76A, Y81A, D105A, and K195A). Importantly, we found that the N116K mutation, but not the E189Q mutation, caused a markedly decreased binding to GAS1, providing a potential explanation for the HPE found associated with the human N115K mutation (Fig. 1*B*). Additionally, the Y81A mutation also significantly disrupted binding to GAS1. Like Asn-116, the Tyr-81 residue is conserved in vertebrate *Shh* genes. We predict that if this mutation were to be observed in a human patient, it would also be associated with phenotypes of defective SHH signaling such as HPE. We have thus identified 2 residues on the SHH A surface that are critical for binding to GAS1.

Based on the above binding data, the prediction that follows is that a decreased GAS1-SHH interaction will correlate with decreased SHH signaling activity. To test this, we employed a cell culture system using NIH3T3 fibroblast cells stably integrated with an SHH-responsive luciferase reporter gene driven by GLI binding sites (NIH3T3-GLI-Luc). This system was previously used to demonstrate the contribution of GAS1 for maximal SHH pathway activation by RNA interference (3). We used a 20 nM concentration of SHH-N-AP, which was determined to be below the saturation threshold for pathway activation (Fig. 2*A*). Consistent with the previous report (12), the SHH D surface mutant had the lowest signaling activity, almost assuredly because it disrupted binding to all tested SHH binding partners (Fig. 2*B*). As predicted for the A surface mutant and its submutants, we found that a change in the activity of a SHH mutant correlates with its change in GAS1 binding. The reduced activity caused by mutation of Asn-116 is consistent with previous reports using a chick neural plate explant assay and C3H10T1/2 cells (23, 24). Our binding results provide an explanation for the observed activity defects and underscore

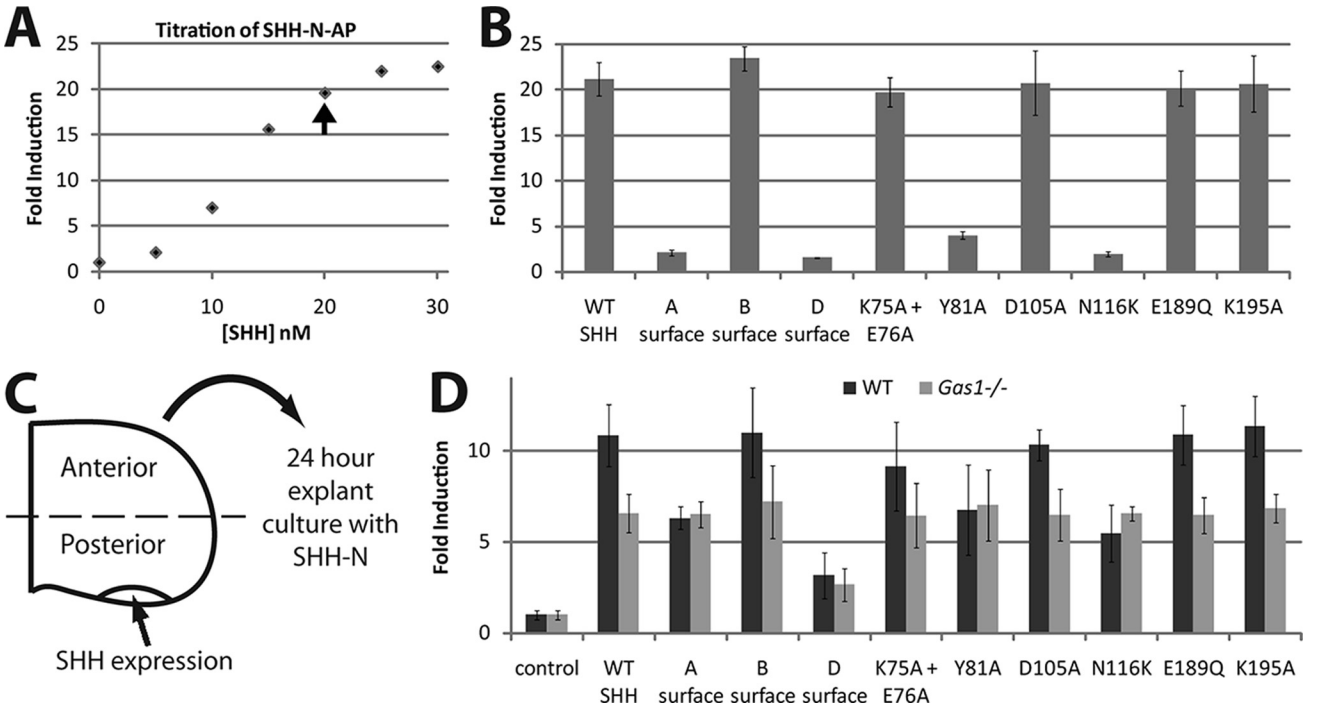

FIGURE 2. **The signaling activity defect of the Y81A and N116K SHH mutants is due to a lack of GAS1 activity.** *A*, titration of WT SHH-N-AP in NIH3T3-GLI-Luc cells; the -fold induction of luciferase activity is shown on the *Y* axis. 20 nM was determined to be the appropriate concentration for subsequent analysis. *B*, various SHH-N-AP mutants tested in NIH3T3-GLI-Luc cells, which are shown on the *X* axis. *C*, diagram depicting how E10.5 forelimb buds were dissected. *D*, qRT-PCR for *Gli1* in WT and *Gas1* mutant limb explants. The *Y* axis shows the -fold induction of *Gli1* transcript level over control explants. Samples were normalized by qRT-PCR for *Gapdh*. All assays were performed at least in triplicate; *error bars* = 1 standard deviation.

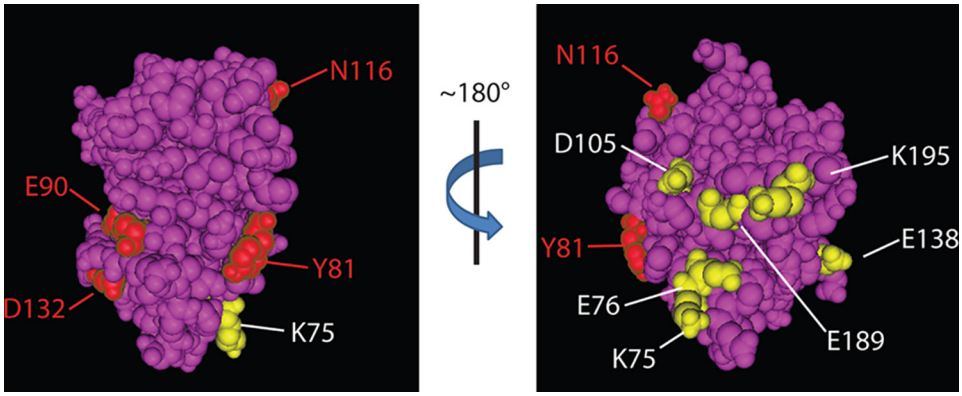

FIGURE 3. **SHH-N structure (from Ref. 17) and specified residues colored using the Cn3D program from NCBI.** Residues colored *red* are important for the SHH-GAS1 interaction. Residues colored *yellow* are likely not part of the GAS1-SHH interface. Two views are shown.

the importance of the SHH signal enhancing activity of GAS1 in this system.

Our data support that the decreased activity of the N116K and Y81A mutants within the A surface of SHH is due to a failure to bind normally to GAS1. However, bacterially produced SHH A surface mutant was shown to have normal activity in a neural plate explant assay (12). It is possible that a cultured neural plate explant does not maintain *Gas1* expression (presumably due to a lack of WNT signaling; see Ref. 5) and thus is not suitable as an assay for the contribution of GAS1. We therefore utilized another established system to assay for SHH activity: cultured limb bud explants, in which we determined that GAS1 levels remain constant for at least 24 h (not shown). Although the explants are not derived from craniofacial tissue, they can serve as an *in vivo* indicator of the significance of the GAS1-SHH interaction. We used the anterior halves of limb buds harvested from WT and *Gas1* mutant E10.5 mouse embryos (Fig. 3*C*). Anterior halves were used because they contain negligible levels of endogenous SHH and *Gas1* expression is high (3, 25). After limb buds were cultured for 24 h in the presence of the various SHH mutants, total RNA was isolated for quantitative real-time reverse transcription-PCR to evaluate expression levels of the SHH target gene *Gli1*. In WT limb buds, the SHH A surface, D surface, Y81A, and N116K mutants all displayed a reduction in signaling activity (Fig. 3*D*), generally consistent with the reduction in activity observed in NIH3T3 cells. The activity defects were more severe in the NIH3T3 cells, which likely reflects a more stringent requirement for GAS1 in that system. As expected in *Gas1* mutant explants, WT SHH-N activity is reduced. Importantly, the SHH A surface, Y81A, and N116K mutants displayed comparable activity in mutant and WT limb buds. This indicates that the reduced activity for these mutant SHH proteins in WT explants is due to a lack of GAS1.

Combining our data, we conclude that the SHH A surface, Y81A, and N116K mutants have defective SHH signaling activity due to their failure to bind to GAS1 normally. Our results can be used to infer that human HPE associated with SHH N115K mutation is most likely due to its reduced GAS1 bind-

ing, hence reduced signaling. 2 of the 3 residues on the SHH D surface (Glu-90 and Asp-132) were shown to be critical for coordinating the binding of calcium to SHH, which is important for the maximal binding of SHH to PTC1, CDO, HIP1, and GAS1 (26). Our findings suggest that the Tyr-81, Glu-90, Asn-116, and Asp-132 residues form part of a contiguous GAS1-SHH binding interface (Fig. 3). The more distantly located other changed residue in surface D (E138A) and the other changed residues on SHH surface A, which do not disrupt GAS1 binding, likely represent a face of SHH that is not involved with ligand binding (Fig. 3). Our work demonstrates an additional point regarding the biochemical mechanism of GAS1 enhancement of SHH signaling; given that SHH appears to have at least a partially unique surface for GAS1 binding, it leaves open the possibility that a ternary complex may form between GAS1, SHH, and PTC1 and that GAS1 may function as a co-receptor for PTC1. Evidence for such a co-receptor model has yet to be obtained.

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