Dual Roles of the Cardin-Weintraub Motif in Multimeric Sonic Hedgehog*

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The fly morphogen Hedgehog (Hh) and its mammalian orthologs, Sonic, Indian, and Desert hedgehog, are secreted signaling molecules that mediate tissue patterning during embryogenesis and function in tissue homeostasis and regeneration in the adult. The function of all Hh family members is regulated at the levels of morphogen multimerization on the surface of producing cells, multimer release, multimer diffusion to target cells, and signal reception. These mechanisms are all known to depend on interactions of positively charged Hh amino acids (the Cardin-Weintraub (CW) motif) with negatively charged heparan sulfate (HS) glycosaminoglycan chains. However, a precise mechanistic understanding of these interactions is still lacking. In this work, we characterized ionic HS interactions of multimeric Sonic hedgehog (called ShhNp) as well as mutant forms lacking one or more CW residues. We found that deletion of all five CW residues as well as site-directed mutagenesis of CW residues Lys33, Arg34, and Lys39 (mouse nomenclature) abolished HS binding. In contrast, CW residues Arg34 and Lys38 did not contribute to HS binding. Analysis and validation of Shh crystal lattice contacts provided an explanation for this finding. We demonstrate that CW residues Arg34 and Lys38 make contact with an acidic groove on the adjacent molecule in the multimer, suggesting a new function of these residues in ShhNp multimerization rather than HS binding. Therefore, the recombinant monomeric morphogen (called ShhN) differs in CW-dependent HS binding and biological activity from physiologically relevant ShhNp multimers, providing new explanations for functional differences observed between ShhN and ShhNp.

The proteins of the Hh family are powerful morphogens that control growth and patterning of developing embryos. Current models for Hh activity suggest that the morphogen disperses from a localized source and forms a gradient that patternsthe fields of responsive cells expressing the Hh receptor Patched (Ptc).

Genetic evidence suggests that this process critically depends on heparan sulfate proteoglycan (HSPG) expression. Upon secretion to the cell surface, Drosophila Hh forms nanoscale oligomers on the cell surface that co-localize with HSPGs (1). HS binds to the Cardin-Weintraub (CW) motif found on all known Hhs and regulates their function in flies (2, 3) and mice (4). In Drosophila (5) as well as in mammalian cell culture (6, 7), Hhs are always released from producing cells in multimeric form, as demonstrated by gel filtration analysis of the soluble morphogen. Release of the multimeric morphogen (the processed Hh N-terminal signaling domain, designated HhNp) from the cell surface depends on the expression of Dispatched (8) and A disintegrin and metalloprotease (ADAM) family members that mediate ectodomain shedding from transfected Bosc23 cells (9). HS is involved in the formation of the HhNp extracellular gradient, which, in the fly, depends on the expression of the Drosophila Exostosin (Ext) family of proteins, encoded by the genes tout velu (ttv), brother of tout velu, and sister of tout velu and the glycosylphosphatidylinositol-linked HSPGs Dally and Dally-like, corresponding to vertebrate glypicans (2, 3, 10). HS expression and Dally-like/glypican expression are also essential for signal reception and modulation on Ptc-expressing expressing cells (10–14) and participate in HhNp-Ihog interaction (15). However, the essential role of direct morphogen-HSPG interactions in embryonic patterning was recently challenged (16). In that report, transgenic mice made deficient in two ShhNp CW amino acid residues implicated in HS binding (17) lacked an Shh-related phenotype, suggesting that direct morphogen-HS interactions were not essential for normal development. However, in that report as well as in others (16–18), CW-dependent HS interactions were characterized using a recombinant, non-physiological monomeric morphogen termed ShhN, whereas embryogenesis depends entirely on the activity of morphogen multimers. Potential variations between ShhN monomer-HS and ShhNp multimer-HS interactions, however, were not investigated.

HS is produced by most cell types in vertebrates and invertebrates. HS biosynthesis (as well as synthesis of heparin, a...
highly sulfated form of HS produced in connective tissue mast cells) occurs in the Golgi compartment on proteoglycan core proteins (19). Exts synthesize the (GlcA1,4GlcNAc1,4)₄ carbohydrate backbone, which is subsequently modified by N-deacetylase/sulfotransferases, O-sulfotransferases, and a GlcA-C5 epimerase. This process results in the generation of specific sulfated protein binding sites (summarized in Ref. 20).

In this work, we compared HS interactions of physiologically relevant multimeric, wild-type, and CW mutant ShhNp with HS interactions of monomeric CW mutant and wild-type forms of ShhN. To this end, we conducted FPLC affinity chromatography employing coupled mouse embryo-derived HS and the various soluble forms of ShhNp and ShhN. We describe that HS elution profiles of multimeric CW mutants differ strikingly from those of the corresponding monomers, indicating that different CW amino acids participate in HS binding of the monomeric and multimeric morphogen. Indeed, we show that two positively charged CW residues that contribute to HS binding of the monomer interact with negatively charged pockets on adjacent molecules in the multimer. These findings shed new light on functional implications of ShhNp multimerization and provide mechanistic and structural insight into biologically relevant ShhNp-HS interactions.

EXPERIMENTAL PROCEDURES

Cloning and Expression of Recombinant Shh—Shh constructs were generated from murine cDNA (NM 009170) by PCR. Secreted, lipidated ShhNp (nucleotides 1–1314, corresponding to amino acids 1–438) was generated in Bosc23 cells (a human 293T derivative), an embryonic kidney cell line routinely used for the expression of lipidated ShhNp (6), and secreted, unlipidated ShhN (nucleotides 1–594, corresponding to amino acids 1–198 of murine Shh) was generated in Bosc23 cells and in Escherichia coli. PCR products were ligated into pGEM (Promega), sequenced, and subsequently released and religated into pcDNA3.1 (Invitrogen) for the expression of secreted, lipidated 19-kDa ShhNp in Bosc23 cells; into pcDNA3.1/myc-HisC (Invitrogen) for the expression of secreted, C-terminally hexa-histidine tagged 28-kDa ShhNHis₆ (the large size due to the presence of a c-Myc and intervening cloning sequence) in Bosc23 cells; into pGEX4T-1 (Amersham Biosciences) for expression of a c-Myc and intervening cloning sequence) in Bosc23 cells and in Escherichia coli. PCR products were ligated into pGEM (Promega), sequenced, and subsequently released and religated into pcDNA3.1. Protein expression was confirmed by Western blotting with anti-CW (Cell Sciences), anti-ShhN (goat IgG; R&D Systems) and polyclonal anti-CW (Cell Signaling Solutions) for secreted and membrane-bound forms of ShhNp. sAP-ShhN using PolyFect (Qiagen). Cells were grown for 36 h, washed with PBS, and incubated in DMEM or serum-free DMEM for various time periods. The serum-free supernatant was then TCA-precipitated, or proteins were enriched by heparin-agarose pull-down.

Protein Purification and Analysis—Proteins were resolved by 15% reducing SDS-PAGE and immunoblotted. Polyclonal anti-ShhN (goat IgG; R&D Systems) and polyclonal anti-CW (Cell Signaling Solutions) were used for primary detection. Visualization was performed after incubation with peroxidase-conjugated donkey anti-goat IgG (detecting anti-ShhN) and goat anti-rabbit IgG (detecting anti-CW) followed by chemiluminescent detection (Pierce). Quantification of Western blotted protein was conducted by ImageJ.

Gel filtration analysis was performed by FPLC (Äkta Protein Purifier (Amersham Biosciences)) using a Superdex200 10/300 GL column (Amersham Biosciences) equilibrated with PBS at 4 °C. Eluted fractions were TCA-precipitated, resolved by 15% SDS-PAGE, and immunoblotted. To determine heparin binding properties of ShhN and ShhNp, the supernatant of transfected Bosc23 cells was subjected to heparin affinity chromatography (Äkta Protein Purifier) using heparin-Sepharose columns (Amersham Biosciences) at 4 °C. Proteins were applied to the columns in the absence of salt, and bound material was eluted with a linear NaCl gradient from 0 to 1.5 M in 0.1 M sodium acetate buffer (pH 6.0). Fractions were precipitated, and eluted proteins were detected immunohistochemically. Shh and ShhNp binding to embryonic tissue HS coupled to NHS-activated Sepharose columns was conducted according to the same protocol. Cleared supernatants of transfected cells, containing the various morphogens, were subjected to automated Äkta affinity purification using a linear salt gradient from 0 to 1 M NaCl in 0.1 M sodium acetate buffer (pH 6.0) for protein elution. sAP-tagged morphogens eluted from HS columns were mixed with 0.1 M glycine buffer, pH 10.4, and directly visualized upon the addition of p-nitrophenolphosphate (Sigma) (Roche Applied Science). sAP quantification was conducted at 405 nm in a spectrophotometer.

Preparation and Analysis of Tissue HS—Embryos were digested overnight with 2 mg/ml Pronase in 320 mM NaCl, 100 mM sodium acetate (pH 5.5) at 40 °C, diluted 1:3 in water, and applied to 2.5 ml of DEAE-Sepacel columns. Glycosaminoglycans were applied to PD-10 (Sephadex G25) columns (Amersham Biosciences). Glycosaminoglycans were lyophilized, Chondroitinase ABC-digested overnight, purified on DEAE as described above, applied to PD-10 columns, and again lyophilized. Via the peptides attached to the HS chains, samples were coupled to NHS-activated Sepharose columns according to the manufacturer’s protocol (Amersham Biosciences). For disaccharide analysis, 10-µg glycosaminoglycan samples were digested using heparin lyases I, II, and III (1.5 milliunits of each in 100-µl reactions) (IBEX, Montreal, Canada) at 37 °C for 1 h, and the resulting disaccharides were separated from undigested material using a 3-kDa spin column (Centricon, Bedford, MA). Compositional disaccharide analysis of cell-derived HS or HS derived from embryos was then carried out by liquid chromatography/mass spectrom-
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et (LC/MS) (21). HS samples were also quantified by this method and/or by carbazole reaction.

For immunohistochemical detection, control slides were digested (IBEX) (50 mM Hepes, 100 mM NaCl, 1 mM CaCl$_2$, 5 μg of BSA/ml, pH 7.0) overnight at 37 °C to prove specificity. sAP-ShhN and mutant forms generated by site-directed mutagenesis were cloned into pWIZ (Gene Therapy Systems, San Diego, CA), expressed in Bosc23 cells, and secreted into the medium. Heparin affinity purification was employed to test for HS binding of the fusion protein, showing elution at 0.9 M salt and thus strong HS interaction. sAP-ShhN-containing medium was adjusted to 0.6 M NaCl to increase binding specificity and was applied to 4% paraformaldehyde-fixed C3H10T1/2 osteoblast precursor cells and frozen embryo sections overnight, followed by three washing steps with PBS. sAP-ShhN bound to HS was directly visualized upon the addition of NBT-BCIP (Roche Applied Science).

**Shh Reporter Assays**—Assays for Hh pathway activation in Shh-LIGHT2 cells, a clonal NIH 3T3 cell line stably incorporating Gli-dependent, firefly luciferase and constitutive Renilla luciferase reporters, were conducted as described (22). Differentiation of C3H10T1/2 osteoblast precursor cells was also used to determine Shh and ShhNp biological activity. 40 h post-transfection, media of ShhNp and mock-transfected BOSC23 cells were harvested. Proteins were first immunoblotted to check for protein release before being used in the subsequent assays. Conditioned media were sterile filtered, mixed with DMEM containing 2% FCS and antibiotics, and applied to C3H10T1/2 cells in 15-mm plates. To some samples, 2.5 μM cycloamine, a specific inhibitor of Shh signaling, was added. Cells were lysed 5 days after induction (20 mM Hepes, 150 mM NaCl, 0.5% Triton X-100, pH 7.4), and alkaline phosphatase (AP) activity was measured at 405 nm after the addition of 120 mM p-nitrophenolphosphate (Sigma) in 0.1 M glycine buffer, pH 10.4. Background differentiation in the absence of exogenous Shh was also quantified. Assays were performed in triplicate.

**Molecular Modeling**—The crystal structure of human Shh (Protein Data Bank entry 3M1N) (23) was displayed and modified employing PyMOL. Crystal contacts were calculated employing CryCo (available on the World Wide Web).

**RESULTS**

**ShhN and ShhNp Binding to Heparin Is Largely CW-independent**—Sequence alignment of Shh N-terminal peptides with various corresponding N termini of vertebrate and invertebrate Hh family members shows that the CW sequence (amino acids 33–39 in the mouse Shh nomenclature; BBBXBB, where B represents basic amino acid residues) (17) is highly conserved and follows a block of conserved residues required for N-terminal acylation (25) (Fig. 1A). Morphogen-HSPG ionic interactions are mediated by binding of positively charged amino acid residues lysine and arginine to sulfated, negatively charged motifs expressed on the HS chains. The three-dimensional structure of human ShhN (Protein Data Bank entry 3M1N) revealed that the N-terminal peptide extended 30 Å away from the globular domain of the protein (23, 26) and that CW residues Arg$^{35}$ and Lys$^{37}$ (human nomenclature; corresponding to residues Arg$^{34}$ and Lys$^{38}$ in the mouse) were located on one side of the extended peptide, whereas Lys$^{32}$, Arg$^{34}$, and Lys$^{38}$ (corresponding to Lys$^{33}$, Arg$^{35}$, and Lys$^{39}$ in the mouse) were located on the other side (Fig. 1B). We thus hypothesized that HS binding of the morphogen would be unlikely to depend equally on all five CW residues. Supporting this idea, N-terminal ShhNp peptides were demonstrated to contribute to morphogen multimerization as well (27). To us, this suggested that monomeric ShhN and multimeric ShhNp may bind to HS differently because some CW residues may contribute to ShhNp protein-protein interactions in the multimer rather than bind to HS. To test this possibility, we generated various monomeric and multimeric mutant forms of murine Shh (supplemental Fig. 1): ShhN$^{ACW}$/ShhNp$^{ACW}$ lacking all CW residues (Lys$^{33}$–Lys$^{39}$), ShhN$^{5x}$A/ShhNp$^{5x}$A via site-directed mutagenesis of all five basic CW residues into alanine residues, ShhN$^{R34A/K38A}$/ShhNp$^{R34A/K38A}$ upon site-directed mutagenesis of residues Arg$^{34}$ and Lys$^{38}$ into alamines (16), and ShhN$^{K33E/R34E}$/ShhNp$^{K33E/R34E}$ upon site-directed mutagenesis of residues Lys$^{33}$ and Arg$^{34}$ into glutamic acid and leucine, respectively.

First, to confirm that CW mutagenesis did not impair the ability of the mutant proteins to multimerize, we analyzed recombinant murine full-length Shh cDNA, resulting in ShhNp, and the monomer control ShhN by gel filtration (Fig. 2A). Gel filtration analysis of media following 2 days of protein expression showed that ShhN was exclusively released in monomeric form in solution, as expected. In contrast, ShhNp
formed multimers exceeding 600 kDa and a second fraction around 300 kDa, consistent with previous reports (28). Despite small differences in the relative abundance of lower and higher molecular weight fractions, all ShhNp mutants multimerized effectively. We thus concluded that all ShhNp mutant forms assemble properly on the cell surface and are exclusively released in multimeric form (29).

How does CW mutagenesis influence morphogen binding to heparin? To answer this question, we conducted heparin affinity chromatography of recombinant, mutant, and wild-type ShhNp as determined by heparin affinity chromatography. All multimeric CW mutant morphogens bind strongly to heparin. Recombinant, soluble ShhNp eluted at high salt concentrations (1–1.3 M NaCl), indicating strong ShhNp–heparin interactions. ShhN, however, showed even higher heparin affinity, eluting at 1.1–1.4 M NaCl. Reduced binding (>0.6 M NaCl) was observed for all mutant forms. C, heparin affinity chromatography of sAP-tagged ShhNp monomers. Equal amounts of the wild-type and mutant morphogens were applied to the column, as assessed by sAP activity determined in the medium. Notably, all morphogens eluted at comparable salt concentrations from heparin. The heparin binding capacity was also comparable; integration of elution curves revealed 6.53, 5.12, 5.57, and 7.1 arbitrary units for sAP-ShhN, sAP-ShhNpK33E/R34L, sAP-ShhNR34A/K38A, and sAP-ShhNp5xA, respectively.

In order to identify functional residues within the ShhN CW motif, sAP-tagged monomeric ShhN and corresponding CW mutants were expressed in Bosc23 cells, and the medium was subjected to heparin affinity chromatography as described above. We found that all monomeric mutant morphogens also bound strongly to heparin, eluting at 0.9–1.3 M NaCl (Fig. 2C). ShhN showed the highest heparin interactions (eluting at 1–1.5 M NaCl), but ShhNp5xA lacking the entire functional CW motif also eluted at 0.9–1.2 M NaCl. This again suggests the presence of a separate Shh heparin binding site. However, heparin represents an artificial substrate, because it is exclusively produced in connective tissue type mast cells and is not known to interact with Shh in vivo.

**ShhN Binding to Embryonic HS Is CW-dependent**—Thus, we next tested Shh binding to physiologically relevant forms of HS, such as mouse embryonic HS. To this end, embryonic day 14 (E14) embryo sections were incubated with equal amounts of recombinant mutant and wild-type ShhNp linked to an sAP tag. Sections were washed, and sAP activity on slides was determined as a readout for the ability of the morphogens to bind HS (Fig. 2D) (16). Slide incubation with sAP-ShhN yielded a strong signal; in contrast, incubation with sAP alone, HS digestion with heparinase I–III prior to sAP-ShhN incubation, or sAP-ShhN incubation in the presence of 1 M NaCl did not yield detectable sAP activity. This demonstrated specific sAP-ShhN binding to HS. Notably, none of the CW mutant monomeric...
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**FIGURE 3. CW mutant ShhN monomers do not bind to HS.** Wild-type and mutant forms of sAP-ShhN were expressed in Bosc23 cells. The supernatants were filtered, and equal sAP activity was applied to columns coupled with E13, E14, and E18 embryonic HS. sAP activity in the flow-through confirmed saturation of Shh binding sites. Bound proteins were then eluted with a linear (0–1 M NaCl) gradient. sAP activity in the eluate was measured, and data were plotted as increased sAP activity over sAP base-line activity prior to elution. A, sAP-ShhN eluted from E13 HS at low (0.6 M) and high (>0.7 M) salt concentrations. In contrast, sAP-ShhN<sub>5xA</sub> and sAP-ShhNR<sub>34A/K38A</sub> showed no detectable binding; little sAP-ShhN<sub>5xR/C38R</sub>, sAP-ShhN<sub>K33E/R34L</sub>, and sAP-ShhN<sub>33K/34R</sub> binding to HS was detected (eluting at 0.5 and 0.5 M NaCl, respectively). B, sAP-ShhN bound to E14 HS, sAP-ShhN<sub>5xA</sub>, sAP-ShhN<sub>C33K/R34L</sub>, and sAP-ShhN<sub>K33E/R34L</sub> showed no detectable binding, and little sAP-ShhN<sub>R34A/K38A</sub> binding to HS was detected (0.53 M NaCl). C, sAP-ShhN elution from E18 HS differed from both previous profiles. The relative amount of sAP-ShhN strong binding sites was increased over weaker binding sites, and a fraction of the wild-type monomer eluted at 0.93 M salt. No sAP-ShhN<sub>5xA</sub>, sAP-ShhN<sub>C33K/R34L</sub>, and sAP-ShhN<sub>K33E/R34L</sub> activity was detected in the eluates, and sAP-ShhN<sub>R34A/K38A</sub> bound only weakly to HS (eluting at 0.53 M NaCl). D, reduced biological activity of ShhN CW mutants. C3H10T1/2 osteoblast precursor cells were incubated with equal amounts of untagged, wild-type, and CW mutant ShhN present in conditioned media, and the relative amount of Shh-induced AP activity was determined as a readout for C3H10T1/2 differentiation and hence for biological activity of the morphogens. Medium obtained from mock-transfected Bosc23 cells was used as a control (mock). Differences in biological activity were always significant (ShhN<sub>5xA</sub>, ShhN<sub>C33K/R34L</sub>, and ShhN<sub>K33E/R34L</sub>; p < 0.001, n = 3; ShhN<sub>K33E/R34L</sub>; p = 0.008, n = 3). Error estimates are standard deviations of the mean.

morphogens bound to HS. This demonstrated that, in contrast to heparin binding, HS binding of ShhN is absolutely dependent on the CW motif, consistent with a previous report (16). Moreover, because morphogens lacking the entire CW motif or just specific residues all failed to bind HS, our data suggest that HS binding depends on total positive charge of the CW motif. This assumption is backed by a report describing high affinity binding of Drosophila HhN to heparin, as determined by surface plasmon resonance, but undetectable binding to HS (18), consistent with the presence of only three basic CW residues (HhN, GRHRARN; murine ShhN, KRRHPKK (17)).

We next tested the interaction of mutant and wild-type sAP-ShhN with various forms of HS by FPLC affinity chromatography. To this end, HS was isolated from E13, E14, and E18 C57/Bl6 mouse embryos and coupled to HiTrap columns. We first established that sAP-Fgf8 and sAP-VEGF elution profiles from all three columns were comparable (supplemental Fig. 2), confirming efficient HS coupling to all three columns. We also confirmed that E18 HS elution profiles of ShhN and N-terminally sAP-tagged sAP-ShhN were comparable (supplemental Fig. 2). Next, equal amounts of wild-type and mutant sAP-ShhN (upon normalization of sAP activity in the input) were applied to each column. sAP activity in the flow-through was also monitored to confirm saturation of ShhN binding sites prior to elution. We detected multiple sAP-ShhN elution peaks from E13, E14, and E18 HS columns, suggesting weak and strong ionic ShhN interactions with HS. Consistent with lower overall HS sulfation if compared with heparin, ShhN eluted from HS columns at salt concentrations ranging from 0.53 to almost 1 M NaCl (Fig. 3, A–C). Weak and strong ionic ShhN interactions with different HS samples were variable, consistent with the observed variation in HS sulfation during development (7) (Table 1). We hypothesized that one possible explanation for the observed strong ionic interactions (0.9–1 M NaCl) would be the presence of “heparin-like,” highly sulfated domains on HS; if correct, we expected that CW mutant forms would also elute from HS columns at ~0.9–1 M salt concentration because of their observed strong interaction with heparin. However, we found elution of only little sAP-ShhN<sub>R34A/K38A</sub> and sAP-ShhN<sub>K33E/R34L</sub> at low salt concentrations and no binding of sAP-ShhN<sub>5xA</sub> and sAP-ShhN<sub>C33K/R34L</sub> to embryonic HS (Fig. 3, A–C) despite comparable sAP-activity in the input and flow-through. This finding shows that the separate heparin binding site does not contribute to the observed strong HS interactions of ShhN.

We tested next whether HS binding of the monomeric morphogen was required for Shh-dependent differentiation of C3H10T1/2 osteoblast precursor cells into AP-producing osteoblasts (30). AP-catalyzed paranitrophenylphosphate conversion into paranitrophenol measured at 405 nm served as a biological readout (Fig. 3D). We first established that media obtained from untransfected cells did not induce differen-
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local concentration of binding sites and larger binding surfaces, and the generation of novel epitopes at the subunit interfaces. Those features may all strongly affect HS binding of the multimer. Using the same HS-coupled FPLC columns, we thus determined binding of multimeric ShhNp and CW mutant forms to different preparations of HS. In this experimental setting, eluted ShhNp was detected by immunoblot analysis and quantified using ImageJ. The strongest signal was then set to 100%, and signals of other fractions were expressed as relative intensities compared with the peak value. As shown in Fig. 4, A and B, ShhNp eluted from E13 HS and E14 HS under high (0.73 m and up) and low (0.47 m) salt conditions, indicating strong and weak HS ionic interactions. This is consistent with the expected structural diversity of the HS mixture extracted from the various embryonic tissues. Ionic interactions of ShhNp with E18 HS were mostly of intermediate strength (around 0.67 m salt) (Fig. 4C). We confirmed these findings at 37 °C (Fig. 4D and inset); here, E18 HS binding of multimeric ShhNp was pronounced, suggesting that ShhNp–HS interactions are temperature-dependent. As expected, ShhNp5xA and ShhNpΔCW did not bind to any HS under the conditions employed. However, in contrast to the monomeric morphogens, substantial E13, E14, and E18 HS binding of ShhNpR34A/K38A and ShhNpΔK33E/R34L was always observed (Figs. 4, A–C). ShhNpR34A/K38A interactions with E13 HS were comparable with those of ShhNp (proteins eluting at 0.47 m and 0.73 m NaCl); in addition, ShhNpΔK33E/R34L elution at intermediate salt conditions was also observed (0.6 m NaCl). A fraction of ShhNpR34A/K38A eluted from E14 HS at even higher salt concentrations than the wild-

### TABLE 1

**HS sulfation during development**

HS was isolated from E13, E14, and E18 embryos, and samples were digested with heparin isoyas. The resulting disaccharides were analyzed by quantitative LC/MS. All HS preparations showed somewhat comparable overall sulfation, E18-derived HS, however, showed reduced relative amounts of mono- and disulfated disaccharides and increased relative amounts of the trisulfated disaccharide D2S6. Values denote the mean percentage of total disaccharide. Disaccharides are abbreviated as follows: D0A0, ΔUA1,4GlcNAc; D0S0, ΔUA1,4Glc; D0A6, ΔUA1,4GlcNac-6S; D0S6, ΔUA1,4GlcNS-6S; D2S0, ΔUA2S1,4Glc; D2S6, ΔUA2S1,4GlcNS-6S.

|        | E13 HS | E14 HS | E18 HS |
|--------|--------|--------|--------|
| D0A0   | 50.3   | 49.15  | 48     |
| D0S0   | 21     | 21.3   | 17     |
| D0A6   | 6      | 5.7    | 6.3    |
| D0S6   | 5.14   | 5.45   | 5.3    |
| D2S0   | 8.7    | 11.3   | 5.7    |
| D2S6 (trisulfated) | 5.12 | 6.6    | 16.5   |
| D2S6 (monosulfated) | 27   | 27     | 23.3   |
| D2S6 (disulfated)   | 13.84 | 16.75  | 11     |
| SO₃⁻/disaccharide   | 0.7   | 0.8    | 0.95   |

### FIGURE 4

**CW mutant ShhNp multimers differ in their HS binding capacities and HS binding strength.** Wild-type and mutant ShhNp were expressed in Bosc23 cells. The supernatant was filtered and applied to the same E13, E14, and E18 embryonic HS-coupled columns, and bound proteins were eluted with salt (0–1 m). ShhNp detected in the eluate was immunoblotted, and signals were quantified by ImageJ. The strongest signal was set to 100%, and signals of other fractions were expressed as relative intensities compared with the peak value. As shown in Fig. 4, A and B, ShhNp eluted from E13 HS and E14 HS under high (0.73 m and up) and low (0.47 m) salt conditions, indicating strong and weak HS ionic interactions. This is consistent with the expected structural diversity of the HS mixture extracted from the various embryonic tissues. Ionic interactions of ShhNp with E18 HS were mostly of intermediate strength (around 0.67 m salt) (Fig. 4C). We confirmed these findings at 37 °C (Fig. 4D and inset); here, E18 HS binding of multimeric ShhNp was pronounced, suggesting that ShhNp–HS interactions are temperature-dependent. As expected, ShhNp5xA and ShhNpΔCW did not bind to any HS under the conditions employed. However, in contrast to the monomeric morphogens, substantial E13, E14, and E18 HS binding of ShhNpR34A/K38A and ShhNpΔK33E/R34L was always observed (Figs. 4, A–C). ShhNpR34A/K38A interactions with E13 HS were comparable with those of ShhNp (proteins eluting at 0.47 m and 0.73 m NaCl); in addition, ShhNpΔK33E/R34L elution at intermediate salt conditions was also observed (0.6 m NaCl). A fraction of ShhNpR34A/K38A eluted from E14 HS at even higher salt concentrations than the wild-

C3H10T1/2 differentiation. Comparable amounts of ShhNpK33E/R34L and ShhNpR34A/K38A, as determined by immunoblotting, showed ~50 and ~90% reduced biological activity, respectively; ShhNp5xA and ShhNpΔCW were inactive. We thus conclude that a fully functional CW motif is essential for ShhN biological activity in this assay.

**CW Residues Contribute Differently to HS Binding of ShhNp and ShhN**—ShhNp multimers must have evolved because of specific advantages over the monomeric form. These advantages may include the possibility of allosteric control, higher...
type form; in contrast, binding to E18 HS was somewhat reduced. From these results, we concluded that CW residues Arg\(^{34}\) and Lys\(^{38}\) contribute to HS binding of monomeric ShhN but not to HS binding of the multimeric morphogen, ShhNp.

In contrast to Fg\(\beta\)8 and VEGF that elute from the various columns at comparable salt concentrations (supplemental Fig. 2), ShhNp elution profiles from E18 HS differed from E13 HS and E14 HS profiles, suggesting that the source of HS determines ShhNp binding. One explanation for the pronounced shift of weak ionic interactions (0.47 – 0.53 m salt) to medium interactions (0.67 m salt) in E18 HS, in addition to substantial strong interactions (eluting at 0.93 m salt), would be the E18-specific expression of a defined ShhNp-specific oligosaccharide binding motif; another possibility would be increased overall E18 HS sulfation. E18 HS disaccharide analysis revealed strongly increased levels of trisulfated disaccharide ΔUA2S1,4GlcNS-6S (16.5% relative abundance in E18 HS versus 5.12% and 6.6% in E13 and E14 HS, respectively) (Table 1). The moderate increase in E18 HS overall sulfation was due to a decrease in relative amounts of monosulfated and disulfated disaccharides. Together, these results indicate that ShhNp may preferentially bind to specifically sulfated HS domains. Moreover, our data suggest the presence of low, medium, and high strength HS binding sites for ShhNp. Last, comparison of ShhN elution profiles (black dashed line) and ShhNp profiles (solid black line) demonstrates different HS binding of multimeric ShhNp and the artificial yet widely employed monomeric morphogen.

**Retained Biological Activity of Selected ShhNp CW Mutants**—Based on their HS binding profiles, we anticipated largely unaltered biological activity of multimeric CW mutants ShhNp\(^{R34A/K38A}\) and ShhNp\(^{K33E/R34L}\), and biological inactivity of CW mutants ShhNp\(^{5xA}\) and ShhNp\(^{ACW}\). To test this idea, we again quantified Shh-dependent differentiation of C3H10T1/2 osteoblast precursor cells (30) (Fig. 5A). Media obtained from untransfected cells did not induce differentiation (mock), and ShhNp effectively induced C3H10T1/2 differentiation (1.65 ± 0.15 arbitrary units, \(n = 3\)). The teratogen cyclopamine (31) completely blocked biological activity of the ShhNp-conditioned media (0.145 ± 0.015, \(p = 0.001, n = 2\)), demonstrating specificity of the assay. As expected, comparable ShhNp\(^{K33E/R34L}\) protein amounts, as determined by immunoblotting, induced C3H10T1/2 differentiation into osteoblasts (1.08 ± 0.1, \(n = 2\)), as did ShhNp\(^{R34A/K38A}\) (1.250 ± 0.05, \(n = 2\)). For all three morphogens, ShhNp, ShhNp\(^{K33E/R34L}\), and ShhNp\(^{R34A/K38A}\), biological activity was always significantly increased if compared with the mock negative control (\(p = 0.0051, p = 0.006,\) and \(p = 0.0023,\) respectively). However, compared with the wild-type morphogen, ShhNp\(^{K33E/R34L}\) and ShhNp\(^{R34A/K38A}\) activities were only insignificantly reduced (\(p = 0.04\) and \(p = 0.12,\) respectively). As expected, ShhNp\(^{5xA}\) and ShhNp\(^{ACW}\) did not induce C3H10T1/2 differentiation. We confirmed that their biological inactivity was not due to misfolding of the mutated morphogens. To this end, we compared total protein levels determined by heparin-Sepharose pull-down with immunoprecipitated ShhNp, employing the confor-

![FIGURE 5. Variable biological activities of ShhNp CW mutants.](image-url)

**FIGURE 5. Variable biological activities of ShhNp CW mutants.** A, C3H10T1/2 osteoblast precursor cells were incubated with wild-type and CW mutant ShhNp conditioned media, and the relative amount of Shh-induced AP activity was determined as a readout for biological activity. Medium obtained from mock-transfected Bosc23 cells was used as a control (mock). ShhNp-induced AP activity was entirely blocked by the teratogen cyclopamine (CA) (2.5 μM, \(p = 0.01, n = 2\)), a specific inhibitor of Shh signaling. Consistent with the previously observed loss of biological activity of monomeric CW-deficient morphogens, ShhNp\(^{5xA}\) and ShhNp\(^{ACW}\) were also inactive in this assay. In contrast, both ShhNp\(^{K33E/R34L}\) and ShhNp\(^{R34A/K38A}\) retained significant biological activities in this assay if compared with the mock control (\(p < 0.001, n = 3\)), consistent with preserved HS binding of the mutant multimeric morphogens. *, \(p = 0.044, n = 3, n.s.,\) not significant (\(p = 0.13, n = 3\)). One representative result of three independent experiments is shown. B, mutant and wild-type morphogens were subjected to immunoprecipitation using the conformation-dependent antibody 5E1 coupled to protein A-Sepharose, and the immune precipitates were subjected to SDS-PAGE and immunoblotting. For normalization of different expression levels, heparin-Sepharose pull-downs of the same expressions served as controls. 5E1 reactivity was detected toward all morphogens, demonstrating that loss of ShhNp\(^{5xA}\) and ShhNp\(^{ACW}\) biological activities was not due to misfolding of the mutated morphogens. C, dose-dependent induction of C3H10T1/2 osteoblast precursor cell differentiation. C3H10T1/2 cells were incubated with equivalent amounts of wild-type or CW mutant ShhNp, and AP activity was measured at different morphogen concentrations as a readout for Shh-induced C3H10T1/2 differentiation. ShhNp\(^{5xA}\) and ShhNp\(^{ACW}\) remained inactive at all concentrations used, and ShhNp, ShhNp\(^{K33E/R34L}\), and ShhNp\(^{R34A/K38A}\) induced C3H10T1/2 cell differentiation in a dose-dependent manner. Cyclopamine was added as a control (2.5 μM).
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FIGURE 6. Intermolecular interactions observed in the human ShhN crystal structure (Protein Data Bank entry 3M1N) suggest protein-protein contacts involving extended N-terminal peptides of symmetry-related molecules in the crystal. One subunit is colored green, the interacting molecule is colored according to electrostatic potential (red, negatively charged; blue, positively charged). A, the N-terminal CW motif interacts with a stretch of negatively charged residues present on the adjacent morphogen in the cluster (right). B and C, detailed view of the interaction site. CW residues Lys33, Arg35, and Lys39 (mouse nomenclature) are surface exposed, whereas residues Arg34 and Lys38 make contact with the negatively charged patch on the adjacent molecule. D, same orientation as C, with the interacting molecule removed for better visualization of residues Arg34 and Lys38.

Dual Functions of CW Residues in Multimeric and Mono-meric Shh—Our results show that deletion of the entire functional CW motif always resulted in the complete loss of HS binding and biological activity of monomeric and multimeric morphogens. In contrast, site-directed mutagenesis of CW residues that mediate HS binding of the monomeric morphogen, such as Arg34 and Lys38 (16), did not abolish HS binding and biological activity of the physiologically relevant, multimeric morphogen. Analysis of the human Shh crystal structure (Protein Data Bank entry 3M1N (23)) provided an explanation for this finding. We observed crystal lattice interactions between N-terminal extensions of ShhN substructures with hydrophobic pockets located on adjacent, symmetry-related Shh molecules (Fig. 6). Here, the basic N-terminal CW motif of one substructure located in close vicinity to a highly negatively charged area consisting of residues Asp88, Glu89, Glu90, Glu126, Asp129, Glu130, Asp131, Glu136, Glu137, and Asp146 on the surface of the symmetry-related substructure (33). Importantly, human Shh CW residues Arg33 and Lys37 (corresponding to residues Arg34 and Lys38 in the mouse) interact with this negatively charged area. CryCo Web analysis also predicted direct contact of residue Arg33 (Arg34 in murine Shh) with the adjacent molecule in the cluster. Residues Lys32, Arg34, and Lys38 (corresponding to Lys33, Arg35, and Lys39 in murine Shh), however, face the opposite, solvent-exposed side of the CW motif. Together, these observations suggested that only murine CW residues Lys33, Arg35, and Lys39 represent potential HS-binding amino acids.

To test this idea, we deleted Lys33, Arg35, and/or Lys39 by site-directed mutagenesis. Gel filtration chromatography of solubilized mutant proteins demonstrated that morphogen multimerization was unaffected, as expected (Fig. 7A), and heparin affinity chromatography confirmed preserved binding of all forms to heparin (Fig. 7B). Subsequent HS affinity chromatography revealed that binding of ShhNpK33A/R35A/K39A to various forms of HS was strongly impaired; in comparison, HS binding of ShhNpK33A/R35A was affected less (Fig. 8, A–C). These results demonstrate that 3M1N crystal contacts accurately predict protein-protein contacts in soluble multimers, supporting a previous study (29). Because HS binding of ShhNpK33A/R35A/K39A to E13 and E18 HS was strongly affected, but ShhNpK33A/R35A binding was affected much less, we further conclude that CW residue Lys39 is most critically involved in the binding of ShhNp to E13 HS and E18 HS.

Based on these findings, we hypothesized that biological activity of ShhNpK33A/R35A/K39A, ShhNpK33A/R35A, and ShhNpK33A/R35A/K39A should be reduced, as observed for ShhNpK33A/R35A and ShhNpK33A/R35A/K39A. However, to our surprise, we found a consistent but insignificant increase in biological activity of ShhNpK33A/R35A/K39A, ShhNpK33A/R35A, and ShhNpK33A/R35A/K39A if tested in C3H10T1/2 differentiation (Fig. 8D) and a significant increase in ShhNpK33A/R35A/K39A and ShhNpK33A/R35A induced firefly luciferase induction in Light2 cells (supplemental Fig. 3). In agreement with previous results (Fig. 5A) (16), ShhNpK33A/R35A-dependent C3H10T1/2 differentiation and firefly luciferase induction in Light2 cells were somewhat reduced (Fig. 8D and supplemental Fig. 3). We thus conclude...
that CW residues Lys\(^{33}\), Arg\(^{35}\), and Lys\(^{39}\) modulate ShhNp biological activity in two cell-based bioassays for Shh activity; however, they are not required for ShhNp activity in these experimental settings.

What is the role of CW residues Arg\(^{34}\) and Lys\(^{38}\)? In this work, we demonstrate that site-directed mutagenesis of these CW residues abolished HS binding of the monomeric morphogen but affected HS binding of the mutant multimer to a lesser degree. 3M1N crystal lattice contacts shown in Fig. 6 and CrystalCo predictions suggested that these residues may instead contribute to ShhNp multimerization (29). To test this possibility, gel filtration chromatography of ShhNp\(^{K33A/R35A/K39A}\), ShhNp\(^{R34A/K38A}\), and ShhNp were conducted. As shown in Fig. 9A, the relative size of ShhNp\(^{R34A/K38A}\) multimers was indeed reduced if compared with those composed of ShhNp\(^{K33A/R35A/K39A}\) and ShhNp, and the relative amount of lower molecular weight multimers was increased. We conclude from this result that CW residues Arg\(^{34}\) and Lys\(^{38}\) contribute to ShhNp multimerization or stabilize the multimeric morphogen in solution. Furthermore, crystal contact analysis and Crys-
talCo calculations predicted Arg34 to be instrumental for N-terminal peptide positioning on the surface of the adjacent subunit in the cluster because of the lack of any upstream ionic interactions, hydrogen bridges or hydrophobic interactions with the adjacent protein. This suggested Arg34-dependent positioning of the otherwise flexible ShhNp N terminus on the surface of the adjacent molecule in the cluster.

To test this idea, we employed the conformation-dependent, monoclonal antibody 5E1 that specifically binds to the ShhNp zinc-coordinating surface epitope that also represents the binding site for the Shh receptor, Ptc (34). This epitope overlaps with the unprocessed N-terminal peptide of an adjacent molecule in the cluster, preventing 5E1 binding of the full-length ShhNp multimer. Upon N-terminal ShhNp processing and removal of the peptide during release (9, 29), however, 5E1 binding is restored. N-terminal processing depends on the presence of a conserved N-terminal cysteine residue serving as an acylation target (Fig. 1); site-directed mutagenesis of cysteine to serine (ShhNpC25S) prevents acylation and subsequent binding (29). Upon N-terminal ShhNp processing and removal of the peptide during release (9, 29), however, 5E1 binding is restored. N-terminal processing depends on the presence of a conserved N-terminal cysteine residue serving as an acylation target (Fig. 1); site-directed mutagenesis of cysteine to serine (ShhNpC25S) prevents acylation and subsequent processing (29). For these reasons, 5E1 binding to soluble morphogen clusters indicates N-terminal truncation during release; in contrast, acylation-deficient, untruncated ShhNpC25S is not 5E1-immunoprecipitated (29). As expected, we detected strong 5E1 reactivity of N-terminally truncated ShhNpSA and blockade of 5E1 binding by retained N-terminal peptides in clusters of unprocessed, full-length ShhNpC25S (Fig. 9B). This result supports the idea that the N terminus of one unprocessed morphogen blocks the 5E1-binding site of an adjacent molecule in the cluster (Fig. 9C, top). We next generated double mutant ShhNpC25S/S24A secreted in unprocessed form, as determined by immunoblotting. However, we observed strong 5E1 reactivity of the full-length morphogen, indicating 5E1 binding to the zinc coordination epitope despite the presence of N-terminal peptides in the cluster. We explain this observation by increased spatial flexibility of the N-terminal peptide in the absence of CW “anchoring” residues (Fig. 9C, bottom) and suggest a new role of CW residues in the positioning of ShhNp N-terminal peptides to the zinc coordination sites of adjacent molecules in the cluster.

**DISCUSSION**

HSPGs have been implicated in Hh release, spreading, and reception, and Shh binds to HS via its CW motif. In this work, we conducted the first biochemical characterization of HS interactions with the multimeric, physiologically relevant morphogen and a systematic analysis of the relative contribution of CW residues to HS binding. We demonstrate that monomeric and multimeric morphogens differ in their capacity to bind HS. Also, CW mutagenesis variably impairs HS binding capabilities of mutant morphogens. All mutant proteins employed in this study were effectively secreted from producing cells, interacted with heparin independent of CW function, and bound the conformation-dependent antibody 5E1, indicating correct folding and Ptc binding capability.

We found that CW residues Arg34 and Lys38 are not involved in HS binding but instead contribute to morphogen multimerization and positioning of the ShhNp N-terminal peptide, as predicted by the 3M1N crystal structure (23). This finding supports the predictive value of 3M1N crystal lattice interactions (29) and aids in interpretation of the observed phenotype of mice made deficient in residues Arg34 and Lys38 (16). In that work, it was demonstrated that HS binding of monomeric ShhNpR34A/K38A was negligible, yet mice made transgenic for this mutation developed normally, except for a specific proliferation phenotype. From these observations, it was concluded that mouse development does not depend on direct Shh-HS interactions. However, monomeric ShhN does not contribute to mouse development, and as shown in our work, HS interactions of multimeric ShhNpR34A/K38A are only moderately affected. We thus suggest that mild phenotypes observed in ShhNpR34A/K38A transgenic mice (16) can be explained by only moderately impaired ShhNpR34A/K38A-HS interactions in vivo.

The observed change in cell proliferation may result from the release of relatively smaller and less active morphogen clusters (6), consistent with our results (supplemental Fig. 4). Smaller ShhNpR34A/K38A multimers may further explain the reduced ShhNpR34A/K38A biological activity detected in C3H10T1/2 cells and Light 2 cells.

In contrast to ShhNpR34A/K38A, we found that HS binding of ShhNpK33A/R35A/K39A was strongly impaired. However, the functional role of CW residues Lys33, Arg35, and Lys39 in cells that receive the Hh signal is less clear. The observed increase in ShhNpK33A/R35A/K39A, ShhNpK33A/R35A, and ShhNpK39A biological activity in C3H10T1/2 and Light 2 cells may be explained by wild-type ShhNp binding to HS present in the serum, in turn competing with Ptc-binding of wild-type but not CW mutant morphogens on receiving cells. This, however, would imply that ShhNp signaling in C3H10T1/2 and Light 2 cells is not strictly dependent on HS expressed on these cells. Consistent with this assumption, disaccharide analysis of C3H10T1/2 cell-derived HS revealed very low relative amounts of trisulfated disaccharide D2S6 and disulfated forms D0S6 and D2S0, resulting in only 0.46 mol of sulfate/disaccharide (supplemental Fig. 5). Unfortunately, insufficient HS amounts isolated from this cell line prevented the generation of HS columns and further direct testing by affinity chromatography. However, three lines of evidence suggest that morphogen binding to C3H10T1/2 HS may be weak, consistent with its low degree of sulfation. First, in comparison with E13 HS, increased E18 HS-ShhNp ionic interactions (Fig. 4) correspond to increased expression of trisulfated disaccharides in E18 HS (Table 1), indicating ShhNp interaction with sulfated HS domains. This is consistent with impaired ShhN binding to low sulfated HS (18) and HS derived from mice made deficient in Ndst1 function (4). HS derived from Ndst1-deficient mouse embryos showed an almost 5-fold relative reduction in the abundance of disulfated D2S0, a 3-fold reduction in monosulfated D0S0, and about 2-fold reduction in trisulfated D2S6. Third, binding of monomeric sAP-ShhN to C3H10T1/2 cells was found to be weak (supplemental Fig. 6; compare with Fig. 2D). Therefore, the low degree of C3H10T1/2 HS sulfation, together with full biological activity of ShhNpK33A/R35A/K39A, challenges the general assumption that Shh signaling on receiving cells is HS-dependent.
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In these cells, HSPG core proteins may instead regulate ShhNp biological functions (13). Instead, morphogen-HS interactions may be more essential in other parts of the Shh pathway. For example, HhNp multimerization in the fly depends on cell surface HSPG expression (1), ShhNp release from producing cells depends on HS-modulated shedding (9), and N-terminal morphogen processing at specific N-terminal sites is essential for biological activation of the solubilized multimer (29). Together, these findings indicate that regulated processing at specific, defined N-terminal peptide positions during release is required for the generation of biologically active morphogens (29) and that this process may be HS-regulated. If correct, biological inactivity of the CW deletion mutant ShhNpACW may thus be caused by the reduced length and aberrant positioning of the N terminus rather than by the lack of HS binding, as discussed above. We confirmed this idea by generating ShhNp-N5 (37), a mutant morphogen lacking the first 5 N-terminal amino acids. This modification resulted in complete loss of biological activity despite the presence of the CW motif (supplemental Fig. 7). Like ShhNp-N5 and ShhNpACW, ShhNpN5A was always secreted in N-terminally truncated form (Fig. 5B). Thus, based on the latter observation, we believe that the CW motif may be required to prevent nonspecific processing during shedding. Because processing at nonspecific, functionally “non-permissive” sites occurs only in the situation in which the spatial flexibility of the N-terminal peptide is increased and HS binding to the CW motif is impaired (in ShhNpA5X, not ShhNpR334A/K38A or ShhNpK333A/R335A/K39A), we suggest that HS binding protects the spatially disturbed N terminus from nonspecific processing.

Last, variable HS affinity of ShhN and ShhNp as described in our work may help explain conflicting results obtained by others when in vivo spreading of monomeric ShhN and multimeric ShhNp were compared. In vertebrates, ShhN showed reduced signaling capabilities and diffusion in comparison with the multimeric wild-type form (6, 38–40), but the opposite situation was found in the limb bud (24) and in the Drosophila wing imaginal disc (41, 42). Thus, based on those reports, it was unclear whether multimerization restricts or promotes morphogen spreading, also because observed differences had been linked to morphogen lipidation rather than multimerization or differential HS binding of monomeric and multimeric morphogens (24, 42, 43). In our work, we demonstrate variable ionic interactions between HS and ShhN or ShhNp and demonstrate that ionic interactions depend on HS sulfation. Thus, we postulate that, depending on the tissue and organism under investigation, ShhNp and ShhN bind to HSPGs differently, in turn affecting extracellular morphogen distribution. Extended ShhNp diffusion may be explained by relatively weaker ShhNp-HSPG interactions if compared with ShhN, as shown in this work, and an extended ShhN gradient by relatively stronger ShhNp-HSPG interactions possibly predominating in other systems or tissues (Fig. 4, compare A and C). If correct, this interpretation illustrates the essential roles that Shh multimerization and CW-dependent ShhNp-HSPG interactions play in morphogen gradient formation.

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