Hedgehog Oligomers*

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Sonic hedgehog (Shh) signaling plays major roles in embryonic development and has also been associated with the progression of certain cancers. Here, Shh family members act directly as long range morphogens, and their ability to do so has been linked to the formation of freely diffusible multimers from the lipidated, cell-tethered monomer (ShhN). In this work we demonstrate that the multimeric morphogen secreted from endogenous sources, such as mouse embryos and primary chick chondrocytes, consists of oligomeric substructures that are "undisruptable" by boiling, denaturants, and reducing agents. Undisruptable (UD) morphogen oligomers vary in molecular weight and possess elevated biological activity if compared with recombinant Sonic hedgehog (ShhN). However, ShhN can also undergo UD oligomerization via a heparan sulfate (HS)-dependent mechanism in vitro, and HS isolated from different sources differs in its ability to mediate UD oligomer formation. Moreover, site-directed mutagenesis of conserved ShhN glutamine residues abolishes UD oligomerization, and inhibitors directed against transglutaminase (TG) activity strongly decrease the amount of chondrocyte-secreted UD oligomers. These findings reveal an unsuspected ability of the N-terminal hedgehog (Hh) signaling domain to form biologically active, covalently cross-linked oligomers and a novel HS function in this TG-catalyzed process. We suggest that in hypertrophic chondrocytes, HS-assisted, TG-mediated Hh oligomerization modulates signaling via enhanced protein signaling activity.

Hedgehog (Hh)4 family members are involved in tissue patterning and progenitor cell proliferation by activation of distinct target genes in a concentration-dependent manner. In vertebrates, three closely related Hh morphogens (Sonic hedgehog (Shh), Indian hedgehog (Ihh), and Desert hedgehog) have been described, and a single ortholog is expressed in Drosophila melanogaster. Production of active morphogen begins with the cleavage of the signal sequence followed by autocatalytic cleavage of the 45-kDa precursor molecule to yield a 19-kDa N-terminal signaling domain. This domain becomes C-terminal-cholesterol-modified during processing and N-terminal-palmitoylated, resulting in a dual-lipidated molecule tightly bound to the surface of producing cells that constitutes the active morphogen (called ShhNp, if derived from the Shh precursor, or IhhNp, if derived from the Ihh precursor) (1). On the (Drosophila) cell surface, lipidated morphogen monomers are organized into suboptical oligomers that are further recruited to preexisting heparan sulfate proteoglycan (HSPG)-rich membrane subdomains to form large, visible multimeric clusters (2). Morphogen release from the cell surface then depends on the expression of Dispatched (3) and ADAM (a disintegrin and metalloprotease) family members. The latter mediate morphogen shedding in an HS-regulated way, as has recently been shown for ShhNp in transfected Bosc23 cells, a HEK 293T-derived cell line (4).

In addition to HS-regulated ShhNp shedding, HS is also involved in the formation of the Hh extracellular gradient, which in the fly depends on the HS co-polymerase Tout-velu (Ttv, exostosin in vertebrates) and the glycosylphosphatidylinositol (GPI)-linked HSPGs Dally and Dally-like, corresponding to vertebrate glypicans (5–7). Exostosins synthesize the HS (GlcA1,4GlcNAc1,4) carbohydrate backbone, which is modified by N-deacetylation/sulfotransferases, O-sulfotransferases, and a GlcA-C5 epimerase. Many growth factors, chemokines, cytokines, and morphogens bind to HS, and the HSPGs are thought to act as co-receptors for these ligands (8). In this work we show a new HS function in hedgehog signaling; that is, the HS-dependent formation of oligomeric substructures stabilized by transglutaminase-catalyzed cross-links.

Eight transglutaminases (TGs) are encoded in the human genome, and at least two have been shown to be secreted forms that participate in extracellular matrix remodeling; they are tissue transglutaminases 2 (TG2) and factor XIIIa (fXIIIa). TGs catalyze covalent e-(γ-glutamyl)lysyl interprotein cross-links in a Ca2+-dependent manner, resulting in the generation of supermolecular structures with extra rigidity and resistance against proteolytic degradation (for review, see Ref. 9). TG activity is involved in the development of the heart, lung, and the central nervous system in addition to salivary gland developing.
opment, blood clotting, and other processes. The two extracellular TGs, TG2 and FXIIa, undergo up-regulation, which is physiological in growth plate hypertrophic chondrocytes and pathological up-regulation in osteoarthritic cartilage (10). TG-mediated cross-linking of in vitro substrates amyloid β-A4 (11), α-synuclein (12), the microtubule-associated Tau protein (13), and myelin basic protein have also been implicated in the pathogenesis of Alzheimer disease, Parkinson disease, and progressive suprabulbar palsy in which the abnormal accumulation of insoluble proteinaceous aggregates causes progressive neuronal death (14). Here, we identified members of the Hh family as new targets for TG cross-linking activity, adding new substrates to the extensive list of TG-cross-linked extracellular proteins.

**EXPERIMENTAL PROCEDURES**

**Cloning and Expression of Recombinant Proteins**—Shh constructs were generated from murine cDNA (NM 009170) using primers carrying desired point mutations or deletions by PCR. In some assays ShhN (resulting in a non-cholesterol-modified but biologically active 19-kDa molecule) was expressed instead of ShhNp (resulting in the biologically active, lipidated 19-kDa morphogen that undergoes multimerization upon secretion to the cell surface) to yield sufficient protein for biochemical analysis. PCR products were subcloned into pGEM (Promega), sequenced, and subsequently cloned into pcDNA3.1/myc-HisC (Invitrogen) for expression in Bosc23 and B16-F1 cells and into pFastBac (Invitrogen) for expression in Sf9 cells and into pGEX4T-1 (Amersham Biosciences) for expression in Bosc23 and B16-F1 cells and into the cell surface to yield sufficient protein for biochemical analysis. PCR products were subcloned into pGEM (Promega), sequenced, and subsequently cloned into pcDNA3.1/myc-HisC (Invitrogen) for expression in Bosc23 and B16-F1 cells and into pFastBac (Invitrogen) for expression in Sf9 cells and into pGEX4T-1 (Amersham Biosciences) for expression in Escherichia coli. A C-terminal HisOH tag was added to some constructs, resulting in the generation of non-lipidated 28-kDa ShhNHisOH (the large size is due to the presence of an additional Myc tag and intervening cloning sequence). Secreted, lipidated ShhNp (nucleotides 1–1314, corresponding to amino acids 1–438) was generated in human Bosc23 cells, and secreted, unlipidated ShhN (nucleotides 1–594, corresponding to amino acids 1–198 of murine Shh) was generated in Bosc23 or mouse melanoma B16-F1 cells. C-terminal-truncated proteins were also generated that were C-terminal-fused to a GPI target sequence derived from human CD55 (containing no glutamine residues, accession number NP 001108224) to allow for the generation of high local concentrations of ShhN peptides on the surface of transfected cells.

**Cell Culture, Protein Purification, and Analysis**—Human Bosc23 and mouse melanoma B16-F1 cells were cultured in DMEM (Invitrogen) supplemented with 10% fetal calf serum (FCS) and 100 μg/ml penicillin/streptomycin and were transfected with plasmids encoding the secreted forms of ShhN and ShhNp using PolyFect (Qiagen). Cells were grown for 36–48 h, and the medium was harvested and ultracentrifuged at 210,000 × g for 60 min to remove proteins bound to membraneous remnants. Proteins were then trichloroacetic acid-precipitated or subjected to heparin-Sepharose (Sigma) pulldown followed by three washing steps in phosphate-buffered saline and analyzed by SDS-PAGE. Where indicated, proteins were not eluted from the heparin beads; instead, the beads were mixed with SDS sample buffer, boiled, and briefly centrifuged, and the sample was loaded onto the gel. Chondrocytes were isolated from the cranial third of 17-day-old chick embryo sterna and cultured in agarose suspension cultures in DMEM supplemented with 100 μg/ml penicillin/streptomycin and 25 μM β-aminopropionitrile, a lysozyme inhibitor, under serum-free conditions in the presence or absence of 100 ng/ml insulin-like growth factor I (IGF-I) or 25 ng/ml 3,5,3′,5′-tetraiodothyronine (thyroxin, T4) for 9–14 days. This resulted in the secretion of endogenous, lipidated hedgehog; as it is unknown whether IhhNp was exclusively produced or whether ShhNp was also present, the chondrocyte-generated Hh is referred to as HhNp. Sf9 cells from the ovarian tissue of Spodoptera frugiperda (German Collection of Microorganisms and Cell Cultures, DSMZ) were grown in Grace’s insect medium (Invitrogen) supplemented with 10% FCS and 10 μg/ml gentamycin. For intracellular expression of ShhN, cells were infected using the Bac-to-Bac baculovirus system (Invitrogen). E. coli BL21 cells (Stratagene) were grown in LB medium containing 100 μg/ml ampicillin. To induce the formation of multimers, proteins were incubated with heparin sodium salt (100 μg/ml, Sigma), chondroitin sulfate sodium salt (100 μg/ml, Sigma), dextran sulfate (100 μg/ml, Sigma), and heparan sulfate fractions isolated from mouse embryos and coupled to Affi-Gel beads (Bio-Rad).

Recombinant ShhN proteins were analyzed by fast protein liquid chromatography (Ákta Protein Purifier (GE Healthcare)) using HisTrap columns for the enrichment of Sf9-expressed proteins or a Superdex200 10/300 GL column for gel filtration analysis equilibrated with phosphate-buffered saline at 4 °C. Eluted fractions were trichloroacetic acid-precipitated before being subjected to SDS-PAGE. Proteins were analyzed by boiling in SDS sample buffer containing 2% SDS, 100 mM dithiothreitol followed by reducing SDS-PAGE and Western blotting. Monoclonal antibody 5E1 that binds to biologically active ShhNp/SHhNp and IhhNp/IIhNp was used for the detection of polyvinylidene difluoride-bound hedgehog as well as to block its biological function in differentiation assays (Developmental Studies Hybridoma Bank, Iowa City, IA). We also used α-ShhN (polyclonal goat IgG; R&D Systems) that detects biologically active and inactive forms of HhNp and HhNp. Tagged proteins were detected by anti-histidine (α-4xh, Qiagen) and anti-Myc (α-Myc, Roche Applied Science) monoclonal antibodies. Secondary detection was performed by incubation with peroxidase-conjugated donkey-α-goat IgG (detecting anti-ShhN) or goat-α-mouse IgG (detecting 5E1, α-4xh and α-Myc, all Dianova) followed by chemiluminescent detection (Pierce).

The following transglutaminase substrates and inhibitors were used: 50 and 100 μM monodansyl cadaverine (N-(5-amino-pentyl)-5-dimethyl aminonaphthalene-1-sulfonamide) (Sigma) and cystamine as well as L-682.777 (1,3,4,5-tetramethyl-2-(2-oxopropyl)thiojimidazolium chloride) (both from Zedira, Germany). Recombinant tissue transglutaminase (rhTG2) was obtained from Zedira and used at 2 μg/ml.

**Preparation and Analysis of Tissue HS**—Tissues or cultured cells 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 Sephadex columns. For disaccharide analysis, proteins attached to the glycosaminoglycans were β-eliminated overnight at 4 °C (0.5 μl NaOH, 1 M
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NaB\textsubscript{4}H\textsubscript{4}, neutralized with acetic acid, and applied to a PD-10 (Sephadex G25) column (GE Healthcare). Glycerosaminoglycans were lyophilized, purified on DEAE as described above, applied to a PD-10 column, and again lyophilized. The samples were then digested using heparin lyases I, II, and III, and the resulting disaccharides were separated from undigested chondroitin sulfate using a 3-kDa spin column (Centricron, Bedford, MA) followed by HPLC analysis using Carborapc PA1 columns (Dionex, Sunnyvale, CA). For the production of HS-beads, HS isolated from mouse embryos at various embryonic (E) stages was Pronase-digested and DEAE-purified as described above and subsequently coupled to AffiGel beads (Bio-Rad) via the HS-associated peptides according to the manufacturer’s instructions.

**Differentiation of C3H10T1/2 Osteoblast Precursor Cells—**C3H10T1/2 cells were grown in DMEM supplemented with FCS and antibiotics as described above. Post-transfection, Shh and mock-transfected Bosc23 cells were cultured in DMEM, 10% FCS for at least 30 h. Conditioned media were then sterile-filtered, mixed 1:1 with Opti-MEM containing 10% FCS and antibiotics, and applied to C3H10T1/2 cells in 15-mm plates. Chondrocyte culture supernatants were sterile-filtered, and FCS was added to a final concentration of 10%, mixed 1:1 with DMEM, 10% FCS containing antibiotics, and subsequently used for differentiation. To some samples 2.5 \textmu M cyclopamine was added, which specifically blocks Hh signaling via binding to the Hh signaling molecule Smoothened (15). Additionally, 1 \mu g/ml 5E1 was added to the medium to inhibit Hh signaling via binding of the morphogen, effectively blocking HhN interaction with its receptor Patched (16, 17). Cells were lysed 5 days after induction (20 m\textmu M Hapes, 150 m\textmu M NaCl, 0.5% Triton X-100, pH 7.4), and alkaline phosphatase activity was measured at 405 nm after the addition of 120 m\textmu M p-nitrophenol (Sigma) in 0.1 m glycine buffer, pH 10.4. Assays were performed in triplicate. Statistical analysis was performed in Excel using Student’s t test (two-tailed, unpaired). All values shown in the text and Figs. 2 and 3 are ±S.D.

**Mass Spectrometric Analysis of ShhN Oligomers—**ShhN was expressed in S9 cells, and the lysate was incubated with heparin-Sepharose beads. The pulled-down morphogen was then applied to SDS-PAGE, and Coomassie-stained bands corresponding to immunoreactive bands were excised. Tryptic digest and ESI-MS/MS analysis followed by MASCOT data base searches identified ShhN peptides in the oligomeric structure. We conducted 2 independent analyses with identical results, resulting in the identification of 13 ShhN peptides (Probability-based Mowse score 200) and also 7 peptides from mouse embryos homogenized in SDS sample buffer or from cultured embryonic cells derived from such embryos. As shown in Fig. 1C, 5E1 immunoblotting revealed the presence of 75-kDa UD oligomers in addition to a 45-Da protein, likely representing the precursor molecule. In contrast, no monomeric morphogen was detected.

**RESULTS**

Chondrocytes Secrete Stable Morphogen Oligomers That Assemble into Higher Order Labile Multimers—Previous studies have demonstrated the presence of ShhNp hexamers that dissociated at high concentrations of salt and detergent (18, 19). In contrast to those “labile” multimers formed from the monomeric morphogen, cultured primary chick chondrocytes endogenously secrete stable morphogen oligomers that were resistant to boiling in the presence of 2% SDS, 100 mM dithiothreitol, and could, thus, clearly be distinguished from the monomer by standard denaturing SDS-PAGE (Fig. 1, A and B). As it is unknown whether IhhNp was exclusively produced or whether ShhNp was also present, the chondrocyte-generated oligomers are referred to as HhNp oligomers. Furthermore, we termed these oligomers undissociatable (UD) HhNp, and the term UD “oligomers” will be used throughout this paper to distinguish these stable forms from the morphogen multimers described previously (18, 19), which do not resist dissociation after treatment with denaturants, including SDS. Immunoblotted UD HhNp oligomers were detected by polyclonal anti-ShhN antibodies as well as by monoclonal, conformation-dependent 5E1 antibodies, the latter being commonly used to detect the biologically active morphogen. The production of UD HhNp oligomers was enhanced by the addition to the medium of 100 ng/ml IGF-I or 25 ng/ml thyroxin (T4). The addition of IGF-I resulted in the generation of the 19-kDa monomer of HhNp as well as three species of UD HhNp-oligomers with apparent molecular masses of about 75-, 120-, or 180-kDa, corresponding to tetrameric, hexameric, or decameric morphogen oligomers, respectively (Fig. 1A). In the presence of T4, the cells generated almost exclusively the 75-kDa species of UD HhNp (Fig. 1B). Under all conditions, however, the 75-kDa HhNp oligomers were the predominant form detected in our assays. Protein aggregation was not a consequence of trichloroacetic acid precipitation or SDS-PAGE because UD oligomers were not detected after trichloroacetic acid precipitation of monomeric ShhNp secreted from transfected human Bosc23 cells. Only ShhNp monomers of 19 kDa were apparent that were also detected upon 5E1 immunoprecipitation (Supplement 1).

Based on previous reports demonstrating the presence of stable ShhNp oligomers in mouse embryos (20), we also analyzed proteins derived from embryonic day (E) 10.5 and E11.5 mouse embryos homogenized in SDS sample buffer or from cultured embryonic cells derived from such embryos. As shown in Fig. 1C, 5E1 immunoblotting revealed the presence of 75-kDa UD oligomers in addition to a 45-Da protein, likely representing the precursor molecule. In contrast, no monomeric morphogen was detected.

We next asked whether UD oligomers additionally assemble into labile higher order multimers comparable to labile ShhNp complexes described by others (18, 19). To answer this question, we fractionated chondrocyte-conditioned medium over a Superdex200 gel filtration column. The various fractions elut-
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FIGURE 1. Endogenously secreted HhNp forms SDS-PAGE-resistant oligomers that form higher order, labile multimers. Immunoblot analysis of chondrocyte-secreted HhNp protein is shown. Chondrocytes isolated from chick sternum were cultured in agarose culture in serum-free medium for 9–14 days. HhNp-containing media were ultracentrifuged, trichloroacetic acid-precipitated, and analyzed by immunoblotting after reducing 12% SDS-PAGE. HhNp proteins were detected with monoclonal 5E1 or polyclonal α-ShhN antibodies, A, in medium harvested from unstimulated chondrocytes (w/o) after 14 days in culture, the α-ShhN antibody 5E1 detected secreted 75-, 120-, and 180-kDa oligomers (arrows). No 19-kDa monomeric HhNp was detectable (arrowhead). The addition of 100 ng/ml IGF-I resulted in the production of 19-kDa HhNp protein and increased generation of 120-kDa oligomers. 5E1 reactivity suggests biological activity of the HhNp oligomers.B, using polyclonal α-ShhN antiserum, UD HhNp oligomers of ~75 kDa were detected upon stimulation with 25 ng/ml thyroxin (T4) after 14 days in culture.

C, immunoblot analysis of embryonic day (E) 10.5 and 11.5 mouse embryo lysates is shown. A 45-kDa morphogen (possibly representing the full-length precursor) as well as 75-kDa oligomers were detected. Morphogen monomers were not detected. D, chondrocytes isolated from chick sternum were cultured in agarose culture in serum-free medium in the presence of T4 for 14 days. Conditioned medium from those cells was then ultracentrifuged and fractionated over a Superdex200 gel filtration column equilibrated in phosphate-buffered saline, and the various fractions were analyzed by immunoblotting after reducing 12% SDS-PAGE to confirm the presence of HhNp multimers. Both polyclonal α-ShhN and monoclonal 5E1 antibodies predominantly detected >600-kDa HhNp multimers that were disrupted into ~75-kDa oligomers upon SDS-PAGE. A minor fraction of 75-kDa oligomers did not form any higher order, labile multimers. Only very low levels of monomeric HhNp and no labile HhNp multimers formed from the 19-kDa monomer were detected (arrowhead).

FIGURE 2. Chondrocyte-expressed HhNp oligomers are SDS/dithiothreitol-resistant and biologically active. Prolonged boiling in reducing Laemmli buffer for 5–60 min (A) and denaturation with sample buffer containing 6 M urea (U) or 6 M guanidine hydrochloride (G.HCl) after the 5 standard minutes of boiling (B) failed to disrupt ~75-kDa UD Hh oligomers (arrow), demonstrating unusual stability of the complexed morphogen. The approximate size of monomeric ShhN is indicated by an arrowhead. C, HhNp oligomers are biologically active. C3H10T1/2 osteoblast precursor cells were incubated for 5 days in chondrocyte-conditioned media in the presence or absence of the teratogen CA that specifically inhibits Hh-driven differentiation into alkaline phosphatase-producing osteoblasts. Conditioned media obtained after 9 and 14 days of chondrocyte culture induced alkaline phosphatase activity that was inhibited by CA co-treatment (2.5 μg/ml, n = 3, p ≤ 0.001) and 5E1 treatment (1 μg/ml, n = 3, p ≤ 0.001). Differentiation of C3H10T1/2 cells is expressed as relative alkaline phosphatase activity of lysed cells after a 5-day induction, measured at 405 nm after the addition of 120 μM p-nitrophenol phosphate.

We next confirmed that chondrocyte-secreted UD oligomers were not residual labile multimers left behind after inadequate denaturation and/or reduction of disulfide-bonds. As shown in Figs. 2, A and B, even prolonged (up to 60 min) boiling in Laemmli buffer or denaturation by boiling for 5 min in sample buffer containing 6 M urea or guanidine hydrochloride failed to disrupt chondrocyte-produced UD HhNp oligomers. To test for the biological activity of these UD oligomers, we took advantage of a sensitive cell-based bioassay, the differentiation of C3H10T1/2 osteoblast precursor cells (21). Conditioned media derived from unstimulated primary chondrocytes after 9 days of culture and 14 days of culture that contained no monomeric HhNp (Fig. 1A, left lane) induced the differentiation of C3H10T1/2 cells into alkaline phosphatase-producing osteoblasts, demonstrating biological activity of the UD oligomers, consistent with their 5E1 reactivity (Fig. 2C). To verify that the C3H10T1/2 differentiation was due to HhNp activity, we used the teratogen cyclopamine (CA), an inhibitor of Hh-dependent signal transduction (15, 22), and the neutralizing anti-Shh antibody 5E1 (16). Indeed, UD multimers consisting of UD oligomers, which probably are stabilized by covalent cross-linking.
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UD HhNp Oligomers Show Enhanced Biological Activity—UD HhNp oligomers must have evolved because of specific advantages over the monomeric form. We, thus, asked whether the biological activity of UD HhNp oligomers was increased if compared with monomeric ShhN, as had been described for the labile multimers formed from ShhNp. To answer this question, we analyzed the supernatants of Bosc23 cells expressing cDNAs encoding hexahistidine-tagged (unlipidated) ShhNHis6 and ShhNp (Fig. 3A) as well as the supernatant of HhNp-secreting chondrocytes by gel filtration chromatography followed by immunoblot analysis (Fig. 3B). Consistent with the findings of others (18, 19), ShhNHis6 only occurred as a monomer in solution. In contrast, ShhNp formed labile multimers consisting of the 19-kDa morphogen and ranging in size from 300 to \(10^3\) kDa. UD HhNp oligomers formed higher order, labile multimers slightly exceeding 600 kDa in size that consisted of 75-kDa UD oligomers. As shown in Fig. 3C, UD HhNp effectively induced the differentiation of C3H10T1/2 cells, whereas approximately equal or even higher amounts of ShhNp and ShhN (Fig. 3B) were biologically less active. In all cases differentiation of C3H10T1/2 cells was strongly impaired by co-treatment with 2.5 \(\mu M\) CA or 1 \(\mu g/ml\) 5E1, confirming that UD oligomers rather than undefined factors in the conditioned media were responsible for the enhanced biological activity.

UD Oligomerization Depends on a Highly Conserved N-terminal Glutamine Residue—To determine the mechanism underlying the formation of stable oligomers from morphogen monomers, we investigated ShhN oligomerization after expression in B16-F1 mouse melanoma cells. To allow for high local protein concentrations on the surface of transfected cells and to target the structures into the same membrane microdomains described for the lipidated morphogen (23), individual peptides were C-terminal-linked with the human CD55 GPI recognition sequence. As shown in Fig. 4A, several truncated Shh peptides readily formed high molecular weight oligomers. The shortest peptide still able to form oligomers (most likely tetramers) in this system ranged from amino acids 25 to 52 (after processing of the signal sequence, amino acids 1–24) (Fig. 4B). Comparison of this amino acid sequence with various corresponding peptides of vertebrate and invertebrate Hhs revealed three notable areas of high sequence conservation; that is, a block of conserved heparin binding Cardin-Weintraub (CW) sequence (amino acids 33–39)(25), and a third block of unknown function, including an absolutely conserved glutamine residue in position 47 (Gln-47). Molecular modeling using the ShhN crystal structure (26) revealed that Gln-47 was situated on the N-terminal, extended domain of the morphogen (Fig. 4C). This location is consistent with Gln-47 serving as a substrate of TG enzymes (27) that recognizes glutamine residues at the surface or, more generally, within terminal extensions protruding from compactly folded protein domains. Thus, we hypothesized this residue to be a TG target, resulting in the formation of \(\epsilon-(\gamma
glutaminyl)lysyl\) cross-links with an undefined lysine residue present on a second protein. Consistent with this idea, a lysine residue adjacent to Gln-47 also is absolutely conserved and always is preceded by uncharged and aliphatic residues. Embedding of lysine residues into such sequences has been reported to enhance their reactivity as TG substrates (28).
However, although Gln-47 was located in Hh proteins within a highly conserved motif consisting of apolar amino acids, which was described to allow for efficient substrate recognition by TG (29), this residue was also followed by a proline residue in position +2. The consensus QXX (whereas X stands for any amino acid) is a poor TG target, at least in gluten peptides (30).

Thus, we first added rTG2 to Sf9-expressed, mutated ShhN lacking all three glutamine residues (Gln-47, -101, and -117, respectively) is a poor TG target, at least in gluten peptides (30).

In Figure 4, A highly conserved Shh N-terminal region is required for the formation of SDS-PAGE-resistant oligomers. A, to determine the linkage site, N-terminal fragments of ShhN were C-terminal-fused to a GPI linker derived from human CD55 and expressed in B16-F1 cells. After processing of the signal sequence, GPI-linked Shh peptides ShhN25–52 (calculated molecular mass, 10.7 kDa), ShhN25–71 (12.7 kDa), ShhN25–91 (15.3 kDa), and ShhN25–126 (21 kDa) were tested for their ability to multimerize by SDS-PAGE and Western blotting. All N-terminal fragments formed stable oligomers on the cell surface, indicating a linkage site in position 25–51. B, shown is a schematic representation of ShhN25–52. Comparison of the amino acid sequences of mammalian N-terminal Hh peptides and *Drosophila* Hh reveals the presence of three highly conserved motifs; that is, a block of conserved residues required for N-terminal acylation (24) ranging from amino acids 25 to 30 (Mouse Shh), the highly conserved heparin binding CW sequence (B, basic residue) (25), and a third block of unknown function including an absolutely conserved glutamine (*) residue. Boxes represent absolutely conserved amino acid residues in at least eight gene products, and gray columns indicate the presence of conserved amino acids. C, shown is a ribbon diagram of ShhN (26). The position of the CW sequence and absolutely conserved residues Lys-46 and Gln-47 is indicated. Both residues are located on the N-terminal-extended peptide. D, ShhN (amino acids 25–198) and a mutant lacking glutamine residues Gln-47, -101, and -117 (ShhN2xQ) were expressed in Sf9 cells. The lysate was incubated with heparin beads and 2 μg/ml tissue transglutaminase 2 (rTG2), pulled down, and analyzed. The mutant protein bound to heparin but, in contrast to the wild type form, did not undergo TG-mediated protein dimerization. E, Sf9 cell lysate after expression of mutant ShhN2xQ lacking glutamine residues Gln-101 and -117 was incubated with heparin beads in the presence or absence of 2 μg/ml rTG2. ShhN2xQ oligomers were detected in the presence of TG2 but not in its absence. F, the addition of 20 mM cystamine (C), a specific inhibitor directed against transglutaminase activity, strongly inhibits aggregation of ShhN25–52. G, cystamine co-treatment also strongly impairs UD oligomerization of endogenous morphogen expressed in primary cells isolated from E11.5 mouse embryos (arrow), *45-kDa Hh precursor.

To directly test for TG-mediated isopeptide bond formation in UD oligomers, we treated GPI-linked ShhN25–52, expressed in B16-F1 cells, with 20 mM cystamine (C), an alternative substrate of several TGs preventing isopeptide cross-link formation. As shown in Figure 4F, protein aggregation was indeed reduced in the presence of the reagent. Next, to demonstrate whether endogenously produced UD oligomers were also linked via TG activity, we incubated cells derived from E11.5 mouse embryos in the presence of 5 and 10 mM cystamine. As expected, treatment resulted in impaired UD oligomer formation in a concentration-dependent manner (Fig. 4G).

We confirmed these results by specific inhibition of chondrocyte HhNp oligomerization. Here, IGF-I-induced formation of UD HhNp oligomers was also abrogated by 20 mM cystamine (Fig. 5A) and reduced by 1–5 μM L-682,777, a specific inhibitor of TG2 and FXIIIa (Fig. 5B) or 50 and 100 μM danyscadaverine (D) (Fig. 5C). In contrast, we found that the addition of 1 mg/ml heparin (Fig. 5D) boosted HhNp oligomerization in IGF-I-stimulated. HhNp-expressing chondrocytes. This was particularly the case for UD HhNp oligomers with molecular masses >75 kDa (Fig. 5D). These data suggest that in some systems such as the Ca^2+^-rich environment in bones, TG and HS secreted from hypertrophic chondrocytes cooperate in the formation of biologically active UD oligomers (Supplement 4).

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recombinant form of ShhN, which was used to confirm the specificity of biologically active ShhN. At this stage, tagged forms of ShhN allowed us to confirm the oligomeric nature of the ShhN protein. Chondrocytes isolated from chick sternum were cultured in agarose culture in serum-free medium for 14 days. HhN-containing media were ultracentrifuged, trichloroacetic acid-precipitated, and analyzed by immunoblotting. A, HhN protein expression in the presence of IGF-I and 20 mM cysteamine (C) resulted in impaired UD oligomerization (arrows) in addition to overall decreased HhNp expression. B, the presence of the TG inhibitor L-682.777 in the medium of HhNp-expressing chondrocytes also resulted in reduced amounts of high molecular weight UD oligomers (arrows). C, HhNp expression in the presence of IGF-I and dapsylcadaverine (D), another TG substrate resulting in abortive transamination, also resulted in reduced amounts of HhNp oligomers in the medium (arrow). D, HhNp expression in the presence of IGF-I and 1 mg/ml heparin (H), however, resulted in increased levels of high molecular weight HhNp oligomers (arrows), indicating a role of sulfated carbohydrate polymers in the process.

FIGURE 6. Heparan sulfate is required for transglutaminase-catalyzed Shh oligomerization. A, ShhN (amino acids 25–198) was expressed intracellularly in SF9 cells (ShhN). ShhN was incubated with heparin beads, and the heparin beads as well as the eluate were analyzed by reducing SDS-PAGE and immunoblotting using polyclonal α-Shh antibody and the monoclonal α-Shh antibody 5E1. Results of duplicate experiments are shown. Heparin-mediated the formation of SDS/dithiothreitol-resistant, >60-kDa ShhN oligomers (arrows) from monomeric ShhN (arrowhead). The identity of oligomeric ShhN was confirmed by nano-LC-ESI-MS/MS of excised bands from the Coomassie-stained gel (arrows). Note the strong reactivity of ShhN oligomers with antibody 5E1, confirming the biological activity of the morphogen oligomer. B, HS was isolated from embryos of various developmental stages, covalently coupled to Affi-Gel, and incubated with hexahistidine-tagged, B16-F1-expressed monomeric ShhNHis6. HS-bound ShhNHis6 was then pulled down, and beads were directly subjected to SDS-PAGE and immunoblotting. Only HS derived from E11.5 embryos induced protein oligomerization, and HS derived from E13.5 and E14.5 embryos bound the monomeric morphogen. C, ShhN and heparin (H) were analyzed by anion exchange HPLC. HS from E11.5 embryos was under-sulfated, resulting in increased levels of non-sulfated (DOA0) and N-sulfated (DO50) disaccharides and a decrease in 6-O-sulfated and 2-O-sulfated disaccharides, especially DOA5 (UA1,4GlcNAc-6S). The relative decrease in 6-O-sulfated and 2-O-sulfated disaccharides, especially DOA5 (UA1,4GlcNAc-6S), D0A6 (UA2S1,4GlcNS), and D0A7 (UA2S1,4GlcNH2-6S), shows a decrease in the biological activity of the morphogen oligomer. D, HS isolated from E11.5–E17.5 embryos, and samples were digested with heparin lyases. The resulting disaccharides were analyzed by anion exchange HPLC. HS from E11.5 embryos was under-sulfated, resulting in increased levels of non-sulfated (DOA0) and N-sulfated (DO50) disaccharides and a decrease in 6-O-sulfated and 2-O-sulfated disaccharides, especially DOA6 and D2A6, if compared with HS derived from E13.5–E17.5 embryos. The relative amount of free amino groups (D2H6 and D2H0), however, was strongly elevated in E11.5 HS. Values denote the mean % of total disaccharide. The abbreviations used denote the following disaccharides: D2H6 (UA1,4GlcNH2-6S), D2H0 (UA2S1,4GlcNH2), DOA0 (UA1,4GlcNAc), DOA6 (UA1,4GlcNAc-6S), D0S0 (UA1,4GlcNS), D2A6 (UA2S1,4GlcNS-6S), DO50 (UA1,4GlcNS-6S), D2A50 (UA2S1,4GlcNS-6S), D2S50 (UA1,4GlcNS-6S), and D2S65 (UA1,4GlcNS-6S).

FIGURE 5. Inhibitors directed against transglutaminase activity impair HhNp aggregation, and heparin results in the increased formation of HhNp oligomers. Chondrocytes isolated from chick sternum were cultured in agarose culture in serum-free medium for 14 days. HhN-containing media were ultracentrifuged, trichloroacetic acid-precipitated, and analyzed by immunoblotting. A, HhNp expression in the presence of IGF-I and 20 mM cysteamine (C) resulted in impaired UD oligomerization (arrows) in addition to overall decreased HhNp expression. B, the presence of the TG inhibitor L-682.777 in the medium of HhNp-expressing chondrocytes also resulted in reduced amounts of high molecular weight UD oligomers (arrows). C, HhNp expression in the presence of IGF-I and dapsylcadaverine (D), another TG substrate resulting in abortive transamination, also resulted in reduced amounts of HhNp oligomers in the medium (arrow). D, HhNp expression in the presence of IGF-I and 1 mg/ml heparin (H), however, resulted in increased levels of high molecular weight HhNp oligomers (arrows), indicating a role of sulfated carbohydrate polymers in the process.

Recombinant, Hexahistidine-tagged 28-kDa ShhNHis6 secreted from B16-F1 cells also formed ~60-kDa ShhNHis6 oligomers. The tryptic peptides were then analyzed by nano-LC-ESI-MS/MS of excised bands from the Coomassie-stained gel (arrows). The identity of oligomeric ShhN was confirmed by nano-LC-ESI-MS/MS of excised bands from the Coomassie-stained gel (arrows). The strong reactivity of ShhN oligomers with antibody 5E1, confirming the biological activity of the morphogen oligomer. B, HS was isolated from embryos of various developmental stages, covalently coupled to Affi-Gel, and incubated with hexahistidine-tagged, B16-F1-expressed monomeric ShhNHis6. HS-bound ShhNHis6 was then pulled down, and beads were directly subjected to SDS-PAGE and immunoblotting. Only HS derived from E11.5 embryos induced protein oligomerization, and HS derived from E13.5 and E14.5 embryos bound the monomeric morphogen. C, ShhN and heparin (H) were analyzed by anion exchange HPLC. HS from E11.5 embryos was under-sulfated, resulting in increased levels of non-sulfated (DOA0) and N-sulfated (DO50) disaccharides and a decrease in 6-O-sulfated and 2-O-sulfated disaccharides, especially DOA6 and D2A6, if compared with HS derived from E13.5–E17.5 embryos. The relative amount of free amino groups (D2H6 and D2H0), however, was strongly elevated in E11.5 HS. Values denote the mean % of total disaccharide. The abbreviations used denote the following disaccharides: D2H6 (UA1,4GlcNH2-6S), D2H0 (UA2S1,4GlcNH2), DOA0 (UA1,4GlcNAc), DOA6 (UA1,4GlcNAc-6S), D0S0 (UA1,4GlcNS), D2A6 (UA2S1,4GlcNS-6S), D2S50 (UA1,4GlcNS-6S), D2S0 (UA2S1,4GlcNS-6S), and D2S65 (UA1,4GlcNS-6S).
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DISCUSSION

The detection of oligomer formation by SDS-PAGE and/or Western blotting and inhibition of protein cross-linking by bifunctional amines is widely used to identify TG protein substrates. In this work we demonstrate by SDS-PAGE the formation of HhNp oligomers starting from the endogenously produced monomers and confirmed the identity of in vitro generated ShhN oligomers by employing two different Hh-specific antibodies in combination with two monoclonal antibodies directed against Myc and hexahistidine tags as well as LC-ESI-MS/MS analysis of in vitro generated ShhN oligomers. Notably, heparin increased HhNp complex formation in chondrocyte cultures, and ShhN effectively formed oligomers in the presence of specific forms of HS. We also made the observation that the formation of large (120 and 180 kDa) oligomers was efficiently inhibited by a range of TG-specific abortive substrates and an inhibitor, however, the 75-kDa form was less affected. Indeed, the incomplete inhibition of UD oligomerization by a range of TG inhibitors is not compatible with the mode of de novo direct polymerization of proteins in a single-phased reaction, which constitutes one type of TG-mediated protein cross-linking reaction. Examples for this type of reaction would be the formation of the vaginal plug after copulation in rodents (31) and lobster hemolymph clotting (32). Notably, another type of known TG action is only incompletely affected by inhibitors or analogue substrates of TGs (9) and can be described as the enzymatic "spot-welding" of non-covalent (e.g. reversible) assemblies. Here, cross-linking occurs in a two-phase system that uses a preformed polymer scaffold, and TG contributes only to the stabilization (maturation) of a preformed reversible polymer structure (9). An example for this second type of TG-mediated reaction is the coagulation of blood. After limited proteolysis of fibrinogen by thrombin, fibrin molecules self-assemble into an array of protofibrils and fibrils, ultimately forming a clot network. The cross-linking enzyme, FXIIIa, then introduces a few strategically located bridges into the preformed, labile polymer to stabilize the clot. Analogous to this process, we suggest that by binding to cell-surface HS chains, membrane-tethered, lipiddated HhNp proteins or preformed nanocomplexes (2) may first assemble into labile multimers similar or identical to those described previously (Fig. 7). In vivo, HhNp protein binding to specifically sulfated HS "scaffolds" may bring Gln-47 and an unidentified lysine residue provided by another monomer into close proximity, allowing for subsequent TG-mediated isopeptide linkage. This will result in UD oligomer formation within labile multimers.

However, although our model would also predict the formation and release of UD oligomeric HhNp from Drosophila cells, surface plasmon resonance studies showed negligible binding of the fly morphogen to HS but strong binding to heparin (33). In contrast, ShhN bound not only to heparin but (although to a lesser extent) also to porcine intestinal HS that was employed in the study. These observations may reflect a strong preference of fly HhNp for highly sulfated regions or forms of HSPGs possibly related to sequence variations within the HS binding CW motif (murine ShhN, GKKRRHPKK; fly HhN, LGHRARN; CW consensus, XBBBXXB, B indicates basic amino acid residues (25)). Alternatively, HS binding of fly HhNp may require specific binding motifs that were likely absent from the porcine HS used in the assay (33) but may be expressed on functional fly HS in vivo. This possibility is supported by the finding that Drosophila HhNp colocalizes with HSPGs in vivo and that fly HhNp/HSPG colocalization was abolished upon deletion of the HS binding CW sequence (2). It is further supported by specific HS requirements for efficient ShhN binding and UD oligomerization in vitro, as shown in this work. The requirement for specific HS motifs may, thus, also explain why ShhN or ShhNp expressed in transfected cell lines were only found as monomers or as labile multimers, in contrast to endogenously expressed HhNp isolated from cultured chondrocytes (19). Possibly, heterologous expression systems lack the specifically sulfated HS isoforms required for UD oligomerization and may...
also lack TG expression, resulting in the generation of only labile morphogen multimers (representing the preformed polymer) (Fig. 7). In contrast, endogenous production of HhNp \textit{in vivo} may be coordinated with specific HS synthesis and TG secretion, resulting in the regulated formation of covalently cross-linked and biologically active UD oligomers within the preformed polymers. For these reasons, the formation of UD oligomers found in the \textit{in vivo} situation was undetectable in previous studies, relying on protein expression in transfected cells. Importantly, TG avidly binds to heparin, but heparin binding only slightly affects TG activity \textit{in vitro} (34). This suggests that UD oligomerization is not merely a result of HS-dependent TG activation.

What may be the function of UD oligomers in specific developmental settings, and what may be their site of expression? Elevated activity of TG2 and fXIIIa have been described as additional features of hypertrophic growth plate chondrocytes (35, 36). Notably, the hypertrophic phenotype can be induced by thyroid hormones in growth plate chondrocytes, and T4 also stimulates TG activity in the extracellular matrix of articular chondrocytes (37). We, thus, suggest that at in the Ca$^{2+}$-rich environment of the developing bone, TG-producing hypertrophic chondrocytes may secrete UD oligomers with specific advantages over the labile multimer or the monomeric form. These advantages may include the possibility of allosteric control, higher local concentration of active sites, larger binding surfaces, and the generation of novel active sites at the subunit interfaces. Those features may all strongly affect the formation of the extracellular morphogen gradient and interaction with the hedgehog receptor Patched. In this work we show enhanced biological activity of UD oligomers and possibly also of labile multimers over the monomeric form. This may be due to the clustering of multiple molecules of the hedgehog receptor Patched, in turn effectively reducing its interaction with Smoothened. Alternatively, increased biological activity of UD oligomers may be explained by increased protein stability in the extracellular matrix due to resistance against proteolytic degradation, consistent with other TG extracellular matrix substrates (9).

In addition to being involved in normal developmental processes, as shown in mouse embryos or hypertrophic chondrocytes isolated from chick embryos, our findings may also play a role in pathophysiological conditions, such as osteoarthritis.
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(OA). Pathologic hypertrophic differentiation also occurs in articular chondrocytes in OA in situ and has the potential to promote OA progression through pathological calcification (38). Both FXIIIα and TG2 are molecular markers of chondrocyte differentiation in the growth plate (35, 39), and TG2 and fXIIIα expression as well as TG activity are up-regulated in human knee OA cartilage chondrocytes (40). Because hypertrophic chondrocytes express TG-linked HhNp oligomers that strongly induced C3H10T1/2 differentiation into osteoblasts in our assays, the findings presented in this work may result in a better understanding of the pathology of OA. In addition to a putative role of UD oligomerization in OA, the novel mechanism suggested here comprising multimerization of Hh proteins by HS scaffold modulation followed by TG-catalyzed cross-linking may also be involved in initiation and progression of neurodegenerative disease. In these scenarios TGs have been implicated in the abnormal accumulation of insoluble and proteinase-resistant proteinaceous aggregates (14). In addition, HS is co-distributed with the abnormal prion protein, PrP(Sc), even in very early disease stages in scrapie-infected mice (41). This raises the possibility that HS may also serve as a scaffold for protein aggregation in these pathophysiological conditions.

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