Domain-specific Modification of Heparan Sulfate by Qsulf1 Modulates the Binding of the Bone Morphogenetic Protein Antagonist Noggin

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We have reported previously that Noggin is a heparin-binding protein and associates with the cell surface through heparan sulfate proteoglycans, where it remains functional for the binding of bone morphogenetic proteins (BMPs). Here we report that the binding of Noggin to the cell surface is highly selective for heparan sulfate and that specific structural features are required for the interaction. Noggin binds most efficiently to heparin sequences composed of 10 or more monosaccharides; N-, 6-O-, and 2-O-sulfates contribute to this interaction. In addition, we have shown that the developmentally regulated endosulfatase Qsulf1 selectively removes sulfate groups from the 6-O position of sugars within the most highly sulfated S domains of heparan sulfate, whereas 6-O-sulfates in the NA/NS domains are not substrates for the enzyme. The activity of Qsulf1 in cells in culture results in the release of Noggin from the cell surface and a restoration of BMP responsiveness to the cells. This shows that Noggin binds to the S domains of heparan sulfate and provides evidence that, in addition to modulating Wnt signaling in vivo by the release of heparan sulfate bound Wnt, Qsulf1 also modulates BMP signaling by the release of surface-bound Noggin.

Heparan sulfate proteoglycans are found ubiquitously both on the surface of cells as well as within the extracellular matrix, where they bind and modify the functions of a diverse array of ligands (1). Loss of function mutations in enzymes of the heparan sulfate biosynthetic pathway have confirmed that this polysaccharide has essential roles during development (8–10). Defects in heparan sulfate structures that modulate Noggin binding to cell surface and a restoration of BMP responsiveness to cells in culture results in the release of Noggin from the cell surface. Here we report an extension of our previous studies that include a determination that Noggin binds to the cell surface selectively through heparan sulfate (HS) in a structurally specific manner. Using heparin as a chemical analogue of HS, we have found that the HS-binding site in Noggin can accommodate up to 10 monosaccharides, and N-, 2-O-, and 6-O-sulfate residues are needed for optimum interaction. Furthermore, we have identified a mechanism by which the occurrence of Noggin-binding sites can be regulated in vivo by the specific action of Qsulf1 on the S domains of HS.

EXPERIMENTAL PROCEDURES

Antibodies—RP57–16 was a gift of Regeneron (Tarrytown, NY). This rat monoclonal antibody was generated by using native human Noggin.

The abbreviations used are: BMP, bone morphogenetic protein; HS, heparan sulfate; DMEM, Dulbecco’s modified Eagle’s medium; CHOK1, acetylated and N-sulfated.

Chinese hamster ovary cells; PBS, phosphate-buffered saline; TBS, Tris-buffered saline; TBST, Tris-buffered saline plus Tween 20; SAX, strong-anion-exchange; HPLC, high pressure liquid chromatography; NAANS, N-acetylated and N-sulfated.

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protein as immunogen. Ascites fluid from SCID mice, affinity-purified by protein G affinity chromatography, was used in Western blotting, immunoprecipitation, and immunofluorescence as indicated below. PhosphoSMAD 1,5,8 was obtained from Cell Signaling Technologies, Inc.

Plasmids—The eukaryotic expression plasmids, pQSUf1 and pMutQSulf1, encoding expression of full-length human QSUf1 and an active-site mutant, respectively, were a gift from C. P. Emerson, Jr. (5).

Cell Culture and Transfection—Chinese hamster ovary cells (CHO-K1) stably expressing Noggin have been described previously (7). Cells were maintained in DMEM/F12 media (BioWhittaker, Inc.) containing 10% fetal bovine serum (HyClone). Liposome-mediated transfection was performed by using Geneporter (Gene Therapy Systems) according to the manufacturer’s recommendations. Stable cell lines previously transfected with Noggin were transfected with plasmids encoding QuSUf1 or MutQSulf1 and selected in DMEM/F12 media containing 10% fetal bovine serum with 200 μg/ml hygromycin, and individual clones were harvested and subcultured. Western blotting with anti-myc monoclonal antibody (9E10) identified positive clones expressing similar levels of wild-type or mutant QuSUf1 enzyme.

Metabolic Labeling, Pulse-chase, and Immunoprecipitation—For metabolic labeling and assessment of Noggin turnover in the cell layer, cells were incubated in methionine- and cysteine-free DMEM (Invitrochem) for 40 min. Trans 𝜆5-S-Label (MP Biomedical) was then added to each well at 200 Ci/ml, and cells were incubated at 37 °C for 30 min. Subsequent to the pulse, cells were washed once with PBS and the media containing 10% fetal bovine serum and chased in the same media. For competition experiments, heparin, heparin fragments, or media containing 10% fetal bovine serum with 200 Ci/ml [35S]Sulfate (ICN Biochemicals) were added during the chase period at 1 μCi/ml.

At the specified time intervals, media was recovered and the cell layers lysed using cold 1% Nonidet P-40, 0.5% deoxycholic acid, 0.1% SDS, 1 mM magnesium chloride, 0.5 mM calcium chloride in phosphate-buffered saline (PBS) containing protease inhibitors of 1 μg/ml pepstatin A, 0.25 mg/ml N-ethylmaleimide, and 0.5 mM phenylmethylsulfonyl fluoride. Media was brought to similar conditions by the addition of concentrated magnesium chloride, 0.5 mM calcium chloride in phosphate-buffered saline. For the assessment of cellular responsiveness to BMP4, live cells grown on glass coverslips were washed twice with warm serum-free medium, incubated with primary antibodies for Noggin (RP57–16 at 4.4 μg/ml), cells were then fixed with 4% paraformaldehyde in PBS at 37 °C, and then washed twice with PBS. The cells were fixed with 4% paraformaldehyde in PBS for 10 min, washed twice with PBS, and then permeabilized in 0.5% Nonidet P-40 for 15 min. The cells were again washed twice with PBS and incubated with secondary antibodies mixed with Oregon Green Phalloidin (Molecular Probes) at 1:200 for 30 min at 22 °C. The cells were washed twice in PBS followed by a water rinse, then mounted in ProLong Antifade (Molecular Probes). Immunofluorescence microscopy was performed with a confocal microscope configured with krypton and UV with the appropriate wavelength filters (568 and 488 nm) for CY3 and Phalloidin excitation.

For the assessment of cellular responsiveness to BMP4, live cells grown on glass coverslips were washed twice and then incubated for 3 h at 37 °C in warm serum-free medium. They were then subsequently washed twice with PBS and incubated with BMP4 at 250 ng/ml for 30 min at 37 °C. Cells were washed two times with PBS, fixed with 4% formaldehyde in PBS for 10 min, washed twice more with PBS, and then permeabilized in 0.2% Triton X-100 for 5 min. After three additional washes with PBS, the cells were blocked by incubating with 10% goat serum and 1% BSA in PBS for 1 h at 22 °C. Cells were incubated overnight at 4 °C with a 1:100 dilution of anti-PhosphoSMAD 1,5,8 (Cell Signaling Technology, Inc.). The cells were then washed three times with PBS and washed with PBS and 0.1% CY3 donkey anti-rabbit at 1:200 for 45 min at 22 °C. After three washes in PBS and a water rinse, the coverslips were then mounted in ProLong Antifade (Molecular Probes) and evaluated by standard immunofluorescence microscopy.

RESULTS

Noggin Binds Selectively to Heparan Sulfate at the Cell Surface—Our previous studies established that Noggin binds to heparin in vitro and to heparan sulfate on the surface of cultured cells (7). In support of the selectivity of this interaction, we performed pulse-chase experiments in which we found that Noggin was displaced from the cell surface by heparin, but not by chondroitin sulfate. In the present studies our goal was to further identify the specificity of the Noggin-glycosaminoglycan interaction at the cell surface. Because heparin is more highly sulfated than chondroitin sulfate and, therefore, has a higher net negative charge, we wanted to see if heparan sulfate with its lower net negative charge, but with similar structural features as heparin, had the same activity. CHO-K1 cells, stably expressing human Noggin, were pulse-labeled with Trans 𝜆5-S-Label for 30 min and then chased in unlabeled media in the presence or absence of glycosaminoglycans, as we have described previously (7). Labeled Noggin was detected in the total cell layer at each time point by immunoprecipitation and SDS-PAGE followed by autoradiography. The
amount of labeled Noggin was quantified and is shown in Fig. 1; it is plotted as the log Noggin recovered as a function of time.

Heparan sulfate derived from porcine mucosa (Fig. 1, HS, open diamonds) had equal activity to heparin (Fig. 1, Heparin, open squares) in the displacement of Noggin from the plasma membrane. By contrast, neither chondroitin sulfate (Fig. 1, CS, open triangles), which has a higher net negative charge than does heparan sulfate, nor dermatan sulfate (Fig. 1, DS, open circles), which contains iduronic acid and is therefore structurally more related to heparan sulfate, had any activity. Similarly, the unsulfated glycosaminoglycan hyaluronic acid (Fig. 1, HA, crossed squares) was unable to displace Noggin from the cell surface. Together, these results imply that Noggin interacts with heparan sulfate in a structurally specific manner and suggested that further experimentation was warranted to elucidate the structural features required for this interaction.

Size Dependence of Noggin Glycosaminoglycan Interactions—To establish the minimum size requirement for the interaction between Noggin and heparan sulfate or heparin, we assessed the ability of various sizes of heparin fragments to displace Noggin from the CHO cell surface. As above, CHOK1 cells stably expressing Noggin were pulse-labeled with Trans-35S-Label in methionine- and cysteine-free media and chased in cold media containing various heparin fragments of defined size, each at 1 μg/ml. Fragments of either 10 saccharides (dp10, □), 12 saccharide (dp12, crossed diamond), or larger (not shown) had equal activity as intact heparin (Heparin, □) in the displacement of surface-bound Noggin.

and the extracellular matrix proteins fibronectin and endostatin (13–15).

Noggin Binding to Heparan Sulfate Depends upon N-, 6-O-, and 2-O-Sulfation—To determine whether Noggin requires specific structural features of the heparan sulfate chain for binding, we repeated similar pulse-chase immunoprecipitation experiments in which we assessed the ability of chemically modified heparin chains to displace cell surface-bound Noggin. Heparin which has been fully desulfated (Fig. 3, open diamonds) shows no activity in the displacement of cell-surface Noggin, confirming the essential requirement for sulfate groups in the binding of Noggin. Similarly, heparin chains which have been either merely N-desulfated (Fig. 3, crossed squares), or N-desulfated and reacetylated (Fig. 3, crossed diamonds) are also inactive, indicating that specifically N-sulfate residues are essential for Noggin binding. Both 6-O (Fig. 3, open triangles) and 2-O-desulfated heparins (Fig. 3, open circles) show a similar significant but partial loss of activity. Although not forming as strong a structural feature of the Noggin binding site, as is apparently the case for N-sulfation, these results suggest that sulfation at 6-O and 2-O positions are also critical for Noggin binding. Together these results imply that Noggin typically binds to the most highly modified domains along the polymer chain of HS.

The Enzyme Qsulf1 Selectively Targets the Catalytic Removal of 6-O-Sulfate Groups within the S Domains of Heparan Sulfate—The enzyme known as Qsulf1 is a developmentally regulated cell-surface protein with strong homology to lysosomal heparan sulfate 6-O-sulfatase. To further evaluate the specificity of this enzyme, stable clones of CHO cells were prepared expressing either the native Qsulf1 or a mutant enzyme bearing conversion of two critical cysteines, which are required for catalytic activity, to alanines (C89A,C90A) (8). The trypsin released heparan sulfate from the surface of CHO cells transfected with either the native Qsulf1 or a mutant enzyme was degraded by heparinases, and the disaccharides were analyzed by SAX-HPLC. The HS from cells harboring the mutated enzyme had a typical HS composition that was composed of near-equal amounts of N-acetylated and N-sulfated units, with 6-O-sulfates present as ΔHexA-GlcNAc,6S, ΔHexA-GlcNS,6S, and ΔHexA,2S-GlcNS,6S. In total, 9% (based on 3H-label) of disaccharides were 6-O-sulfated (Fig. 4). The HS isolated from cells
Qsulf1 Activity Releases Cell Surface-bound Noggin—Our results above indicated that 6-O-, 2-O-, and N-sulfate residues all participated in the binding of Noggin to heparan sulfate. This suggested that Noggin might actually bind to the S domains of heparan sulfate where the trisulfated disaccharides (HexA2S-GlcNS,6S) reside. Because Qsulf1 seems to selectively target these regions of the heparan sulfate chain, we reasoned that Qsulf1 might modulate cell-surface binding of Noggin. To assess this possibility, the CHO cells stably expressing Noggin, used in the studies above, were subsequently transfected with either Qsulf1 or the mutant enzyme and selected for stable expression.

As described above, pulse-chase analysis of these cells was performed, followed by immunoprecipitation of cell layer-associated Noggin and its quantification after PAGE-gel electrophoresis. Cells expressing active Qsulf1 (Fig. 7, diamonds) had significantly increased clearance of Noggin from the cell layer when compared with control cells (Fig. 7, triangles) not expressing enzyme. Cells expressing MutQsulf1 (Fig. 1, circles) in which the catalytic site of the enzyme has been mutated revealed identical pulse-chase kinetics as control cells. This altered kinetics of Noggin release results in dramatically altered steady-state levels of Noggin on the cell surface, as detected by immunohistochemistry of fixed, non-permeabilized cells.

As we have described previously, CHO cells expressing Noggin display abundant punctate localization of this protein at
the cell surface, where it is bound by means of heparan sulfate (Fig. 8A). In contrast, cells simultaneously expressing Qsulf1 show a dramatic reduction in cell-surface Noggin (Fig. 8G). A similar reduction in level of cell-surface Noggin was detected (Fig. 8E) even with lower levels of Qsulf1 expression (Fig. 8K). Only at extremely low levels of Qsulf1 expression (Fig. 8K) did cell-surface staining return to near-control levels. As in the case of the pulse-chase analysis, loss of cell-surface localization depended upon intact enzyme activity, as shown by co-expression of mutated Qsulf1 (Fig. 8I).

Release of Cell-surface Noggin by Qsulf1 Activity Results in BMP4 Responsiveness—In our previous studies we have shown that Noggin, bound to the cell surface through an interaction with heparan sulfate, remains functional at that location in the binding of BMP4. In our present study we have shown that Qsulf1 catalytic activity results in the release of this Noggin from the plasma membrane. Therefore, we would predict that the activity of Qsulf1 functions to release the localized BMP inhibition activity of Noggin near the plasma membrane, thus resulting in an increased accessibility of BMP to its signaling receptor at the plasma membrane and an increased BMP responsiveness of the cell.

To access this, QS3-NG and QMut-NG cells (Fig. 8) were incubated in serum-free medium and treated with BMP4 in culture. After stimulation with BMP4, cells were permeabilized and their responsiveness was accessed by the detection of phosphoSMAD 1,5,8 using immunofluorescence. QMut-NG cells expressing the catalytic site mutant of Qsulf1 have, as shown above, abundant plasma membrane localization of Noggin; consistent with this, they fail to respond to exogenous BMP4 as indicated by the lack of SMAD phosphorylation (Fig. 9A).
contrast, expression of active Qsulf1 results as shown above in the near-quantitative release of cell-surface Noggin. This results in a release of membrane-associated BMP inhibitory activity that leads to the restoration of BMP4 responsiveness of these cells, as can be seen by the detection of phosphorylated SMAD in the nucleus (Fig. 9C).

**DISCUSSION**

Patterning events during development are determined by both the short- and long-range action of signaling molecules that regulate cell fate. One important class of these signaling molecules is the family of BMPs (16, 17), which specify cell fate in response to the local level of BMP activity in the immediate environment of the cell (18–20). Secreted proteins, such as Noggin, act as antagonists of BMP function by binding BMPs and preventing their interactions with BMP receptors at the cell surface (21, 22). Thus antagonists like Noggin can function to regulate patterning events by helping to shape activity gradients of BMPs in vivo. We have reported previously that Noggin release and diffusion from the cell surface depends upon heparan sulfate (7). Noggin binds tightly to the cell surface through heparan sulfate proteoglycans and can be directly internalized and degraded at the plasma membrane without significant diffusion. Therefore, we have proposed that heparan sulfate proteoglycans might regulate BMP-dependent patterning events by controlling the range and duration of action of Noggin in vivo (7).

Our current findings highlight the fact that the binding of Noggin to the cell surface is specific to heparan sulfate and that there are certain structural characteristics essential to the Noggin binding site. The minimal Noggin binding site seems to accommodate a length of 10 monosaccharides and has an essential requirement for N-sulfate groups, as well as a relative requirement for 6-O- and 2-O-sulfate, suggesting that Noggin binding sites contain tri-sulfated disaccharides. The identification that Noggin binding to heparan sulfate has specific structural requirements supports the hypothesis that Noggin, like many other heparin-binding proteins, may bind selectively in vivo to relatively specific sequences of heparan sulfate. Although heparan sulfate is ubiquitous in nature, its specific structure is extremely heterogeneous. There is mounting evidence that the structure of heparan sulfate chains found in vivo may be both spatially and temporally regulated to impart tissue-specific preferences for the functional binding of particular ligands (23–25). How this structural diversity is generated is complex. There are multiple isoforms for many of the key enzymes in the biosynthesis of heparan sulfate. The developmentally regulated expression of these different isoforms is likely to contribute to some of the diversity found within heparan sulfate structures in vivo. In addition, it has been recently appreciated that there are membrane-associated sulfatases capable of editing heparan sulfate sequences in vivo and apparently, therefore, modifying heparan sulfate functions. One such enzyme is Qsulf1 (8–10).

Qsulf1 was originally cloned as a Sonic hedgehog-responsive gene (Shh) activated during somite formation and was recognized to have homology to the lysosomal N-acetylgalosamine sulfatases and therefore speculated to be a heparan sulfate 6-O-sulfatase (8), an activity recently confirmed (9, 10). Blockage of Qsulf1 function in vivo through the use of antisense phosphorothiolated oligonucleotides resulted specifically in the inhibition of Myf5 expression, a Wnt-induced gene, in the epaxial somite muscle progenitor cells (8). Qsulf1 has been shown in vitro to directly regulate Wnt1 signaling, apparently by modification of heparan sulfate chains to a lower affinity binding state for Wnt1 (9). This is speculated to

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**Fig. 7.** Catalytic activity of Qsulf1 is associated with more rapid release of Noggin from the cell surface. CHO1 cells stably expressing Noggin, as well as either active Qsulf1 or the inactive catalytic site mutant MutQSulf1 were labeled with Trans-35S-Label in methionine- and cysteine-free media and chased in cold media. Cells expressing Qsulf1 (○) had a significantly reduced half-life of Noggin at the cell surface, whereas cells expressing MutQSulf1 (□) were indistinguishable from control (△).

**Fig. 8.** Qsulf1 activity results in the reduction in steady-state levels of Noggin at the cell surface. Double immunofluorescence images of CHO1 cells stably expressing Noggin and various levels of Qsulf1 or the catalytic mutant QMut, using anti-Noggin antibodies (A, C, E, G, and I) and phalloidin (B, D, F, H, and J). A, control cells expressing Noggin display abundant punctate localization of Noggin at the cell surface. Cells expressing progressively increasing levels of active Qsulf1 as detected by Western blotting (K) reveal reduced amounts of cell surface-associated Noggin (C, E, and G). By contrast, levels of cell-surface Noggin were unchanged in cells expressing the catalytic site mutant enzyme QMut-NG (I).
result in the release of Wnt1 and its increased availability for signaling.

Our results suggest that Qsulf1 might have a more complex relationship to the control of patterning events during development. Qsulf1 expression in the medial somite is likely to have important in vivo functions in the regulation of Noggin activity as well. Noggin is expressed in the dorsomedial lip of the developing somite. Antagonism of BMP4, arising from the lateral plate mesoderm, is thought to be a function of Noggin, which contributes with Wnt to the specification of epaxial musculature in the medial somite (26–28). Coincident expression of Qsulf1 in the medial somite would be expected, based upon our in vitro data, also to result in reduced binding of Noggin to heparan sulfate proteoglycans on the surface of medial somitic tissue. This would be expected to lead to more effective diffusion of Noggin within this tissue, whereas diffusion of Noggin into the adjacent lateral somite would likely be restricted by heparan sulfate on the surface of these cells with higher affinity binding, thus helping to establish a limit on Noggin action and defining the boundary between the lateral and medial somite. Qsulf1 could play a similar role in the release of Noggin from the surface of cells within the notochord, where both of these proteins are also co-expressed and, therefore, have other functions with respect to BMP antagonism in neural patterning.

The discovery that the catalytic activity of Qsulf1 modifies the cell-surface binding of components of at least two developmentally significant signaling pathways suggests that this has evolved as a general mechanism to regulate cellular responsiveness to perhaps many heparin-binding growth factors. Two proteins related to Qsulf1 have been identified in humans: Hsulf-1, which seems to be the ortholog of Qsulf-1, and Hsulf-2. Each had a distinct pattern of expression in adult tissues (10). These enzymes have very similar activities on heparin substrates, but it remains to be determined whether their actions on heparan sulfate can be distinguished in ways that might reflect functional divergence.

Our analysis of the composition of heparan sulfate chains after exposure to Qsulf1 has revealed a detail previously not fully appreciated. The activity of Qsulf1 seems to be almost exclusively targeted toward the highly sulfated S domains of the heparan sulfate chain, while leaving the NANA domains virtually unchanged. This is clear from disaccharide analyses of Qsulf1 transfectants which have a marked reduction (~80%) in the level of the trisulfated disaccharide HexA,2S-GlCN,6S, which is found almost exclusively in the S domains (11). However, we consistently found that there was always a small proportion of this disaccharide that resisted Qsulf1 action. The 6-O-sulfate in the NA/NS domains, where the HexA-GlcNAc,6S unit is present together with HexA-GlcNS,6S (29, 30), were not affected by Qsulf1. Taking into consideration the sequence and sulfation patterns of the S domains in HS, these findings suggest that Qsulf1 recognizes the 6-O-sulfate groups in regions where the substituted GlcNS residue is flanked by idurionate-2-sulfate (i.e., IdOa,2S-GlCN,6S,IdoA,2S). On the other hand, 6-O-sulfated sequences in the NA/NS domains, which take the form of GlCN/IdoA-GlcNAc±6S-GlcA-GlcNS±6S are unaffected (Figs. 4 and 6). Thus, Qsulf1 has an “editing” effect on the 6-sulfation pattern of heparan sulfate; this targeted Qsulf1-mediated 6-O-desulfation of HS clearly impairs Noggin and Wnt/Wg binding, indicating that these proteins interact with the S domain in HS. Moreover, we can also predict that it will suppress signaling induced by FGF1 and FGF2, which also requires S domain 6-O-sulfation (31, 32). It remains to be seen whether these two growth factors are co-expressed with Qsulf1 in developing tissues. In summary, we have shown that Noggin binding to heparan sulfate depends upon 6-sulfate groups being present in the S domains. Qsulf1 impairs Noggin binding by specifically removing these sulfate groups, without affecting 6-O-sulfates in the NA/NS domains. Thus, Qsulf1 subtly changes the sulfation pattern of heparan sulfate on the cell surface, “tuning” its bioactivities to its key functions in the embryo of regulating the actions of growth factors and morphogens at different stages of development. Further molecular and genetic studies will be helpful in ascertaining the full significance of Qsulf1 to biological processes in vivo.

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