Heparan Sulfate Proteoglycans Retain Noggin at the Cell Surface:
A Potential Mechanism for Shaping BMP Gradients

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Abstract

Bone morphogenetic proteins (BMPs) are expressed broadly and regulate a diverse array of developmental events \textit{in vivo}. Essential to many of these functions is the establishment of activity gradients of BMP, which provide positional information that influences cell fates. Secreted polypeptides, such as Noggin, bind BMPs and inhibit their function by preventing interaction with receptors on the cell surface. These BMP antagonists are assumed to be diffusible, and therefore potentially important in the establishment of BMP activity gradients \textit{in vivo}. Nothing is known, however, about the potential interactions between Noggin and components of the cell surface or extracellular matrix that might limit its diffusion. We have found that Noggin binds strongly to heparin \textit{in vitro}, and to heparan sulfate proteoglycans on the surface of culture cells. Noggin is detected only on the surface of cells that express heparan sulfate, can be specifically displaced from cells by heparin, and can be directly crosslinked to a cell surface proteoglycan in culture. Heparan sulfate bound Noggin remains functional and can bind BMP4 at the plasma membrane. A Noggin mutant with a deletion in a putative heparin binding domain has reduced binding to heparin and does not bind to the cell surface, but has preserved BMP binding and antagonist functions. Our results imply that interactions between Noggin and heparan sulfate proteoglycans \textit{in vivo} regulate diffusion, and therefore the formation of gradients of BMP activity.
Introduction

The gene noggin encodes a member of one of at least four distinct gene families encoding secreted polypeptides that bind to members of the TGF-β superfamily, such as BMP4, and inhibit the function of these signaling proteins by preventing their interaction with receptors on the cell surface (1,2). Other antagonists with related functions include Chordin, Follistatin, and members of the DAN family (1,3-6). Although structurally distinct, members of these gene families have in some cases similar ligand specificity, overlapping patterns of expression, and in the case of Chordin and Noggin these proteins apparently are capable of at least partial compensation for each other, for example during forebrain development in the mouse (7). In addition to these multiple secreted BMP antagonists, there are other secreted proteins whose primary function is to overcome this antagonism (8,9). Thus, there is a highly complex system to regulate the bioavailability and consequently the activities of BMPs in the extracellular space.

Members of the BMP gene family are broadly expressed and their functions have been implicated in a wide range of developmental processes (10,11). In most situations, BMPs appear to function as morphogens (12-14). Morphogens function over long ranges through the formation of gradients of activity. Cells sense the strength of the morphogen signal across this gradient and respond by induction of dose dependent patterns of gene activation, which then serve to specify cell fate. Despite evidence for gradients of BMP activity in many tissues, there is no evidence that bmp4 transcripts have graded expression patterns in many of these same tissues (15-17). This has led to the suggestion that post-translational mechanisms are responsible for the establishment of BMP activity gradients \textit{in vivo} (12,14,18). Specifically, it has been hypothesized that the diffusion of a secreted BMP antagonist from organizer regions...
might establish gradients of BMP antagonism, thereby resulting in inverse activity gradients of BMP. Therefore, BMP antagonists themselves may behave as morphogens. Blitz et al. have recently demonstrated that when Chordin is expressed at high doses by microinjection of mRNA it can directly function to inhibit BMP over large distances in the Xenopus embryo and can apparently establish a BMP activity gradient over several cell diameters (19). It remains untested, however, whether endogenous Chordin similarly acts over long distances during normal development (19).

Testing of this hypothesis is restricted to indirect interpretations of the range of BMP antagonist function since adequate reagents do not exist to localize the precise physical range of any BMP antagonists in vivo in relation to their site of production. Accurately predicting this physical range is dependent on understanding the potential interactions between BMP antagonists and components of the cell surface and the extracellular matrix in vivo. Little is known about these potential properties, and typically it has been assumed that these proteins are in fact freely diffusible following secretion. Heparan sulfate proteoglycans are found abundantly on the surface of all adherent cells and within the extracellular matrix where they bind and regulate the functions of a wide range of ligands (20). More importantly, members of the glypican family of heparan sulfate proteoglycans have been found to specifically modify cellular responsiveness to BMPs in vivo (21,22). We therefore hypothesized that heparan sulfate proteoglycans in vivo might regulate BMP function through interactions with BMP antagonists. In this study we have evaluated the ability of heparan sulfate proteoglycans to bind to one well characterized BMP antagonist, Noggin, and to influence its cellular localization in cultured cells. We report that Noggin binds strongly to heparin-Sepharose in vitro and to heparan sulfate
proteoglycans on the surface of cultured cells, thereby localizing Noggin to the cell surface. This bound Noggin remains functional and indeed can bind BMP4 to the cell surface. Genetically engineered mutant Noggin proteins bearing deletions in a putative heparin-binding domain significantly reduce Noggin’s ability to bind heparin (23) and eliminate binding to the cell surface. Furthermore, these mutations have been shown to have little effect on the function of Noggin as a BMP antagonist (23) and are consistent with the crystal structure of the Noggin/BMP-7 complex which shows that the heparin binding site lies in a separate domain from that which binds BMP (Jay Groppe, Jason Greenwald, Aris N. Economides, Markus Affolter, and Senyon Choe, in preparation). These results suggest that it is likely that interactions between Noggin and heparan sulfate proteoglycans in vivo play a significant role in the physical range of Noggin actions.

Experimental Procedures

Antibodies- RP57-16, RP57-21, and 57-06 were a gift of Regeneron. These rat monoclonal antibodies were generated using native human noggin 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.

Plasmids- The eukaryotic expression plasmids, pSRα.hNog and pSRα.hNogΔB2, encoding expression of full-length human Noggin (hNog) and a genetically engineered deletion mutant were a gift of Regeneron. For the generation of stably transfected cell lines, cells were co-
transfected with pSVZeo from Invitrogen.

**Cell Culture and Transfection**- Parental Chinese Hamster Ovary cells (CHOK1) as well as mutant lines derived from these same cells, but which are defective in either heparan sulfate (PGSD-677) or both heparan and chondroitin sulfate (PGSA-745) biosynthesis, were a gift of Jeff Esko (UC San Diego, CA). Cells were maintained in DMEM/F12 media (BioWhittaker) containing 10% fetal bovine serum (Hyclone). Liposome mediated transfection was performed using Geneporter (Gene Therapy Systems) according to the manufacturers recommendations. Cells were cotransfected with pSVZeo (Invitrogen, San Diego, CA), selected with 0.5 mg/ml Zeocin (Invitrogen, San Diego, CA) in DMEM/F12 media containing 10% fetal bovine serum, and individual clones harvested and subcultured. Western blotting with RP57-16 antibody identified positive clones expressing similar levels of Noggin.

**Metabolic Labeling, Pulse Chase and Immunoprecipitation**- For metabolic labeling of Noggin expressing cultures, cells were incubated in methionine- and cysteine-free DMEM (Life Technologies, Inc.) for 40 min. Translabel-35S (ICN) was then added to each well at 200 µCi/ml, and cells incubated at 37°C for 30 minutes. Subsequent to this pulse, cells were washed once with DMEM/F12 media containing 10% fetal bovine serum, and chased in the same media. For competition experiments heparin or chondroitin sulfate (Sigma, St. Louis, MO) were added during the chase period at 1 µg/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 addition of concentrated buffer. Cell debris was removed from both cell layer extracts and media samples by centrifugation at 14,000 rpm for 1 min. Supernatants were then precleared by the addition of rat IgG (Sigma, St. Louis, MO) at 1 µg/ml, incubated for 30 min at 4°C, followed by addition of Protein-G Sepharose and incubation overnight at 4°C. Samples were then centrifuged and the supernatants used for immunoprecipitation.

RP57-16 (Regeneron, Tarrytown, N.Y.), was added to each sample at 1 µg/ml and incubated for 1 h at 4°C. Protein-G Sepharose was subsequently added and incubated for an additional 1 h. Beads were washed twice with the original lysis buffer without protease inhibitors and then twice with PBS containing magnesium and calcium chloride. Immunoprecipitated products were recovered from the beads by boiling in SDS-PAGE sample buffer and analyzed by electrophoresis on 10-20% gradient SDS-PAGE gels (BioRad, Richmond, CA), detecting immunoprecipitated products by autoradiography or PhosphorImager.

**Western Blot Analysis** - Conditioned media and extracts from cells expressing human noggin, as well as fractions separated by affinity chromatography of Noggin on heparin-Sepharose, were electrophoresed on 10-20% gradient SDS-PAGE gels (BioRad), and transferred to Zetaprobe (BioRad) by electoblotting. Filters were blocked for 1 h at room temperature using 5% non-fat dry milk (NFDM) in 20 mM Tris-HCl, pH 7.4, 150 mM sodium chloride (TBS), plus 0.1% Tween 20 (TBST). Blocked filters were probed with RP57-16 at 20 ng/ml in 2.5% NFDM in
TBST for 1 h at room temperature. Following three washes with TBST, incubation with an anti-rat HRP-conjugated secondary antibody in TBST for 1 h, and three remaining washes with TBST, protein bands were detected by ECL.

**Inorganic Sulfate Labeling, Crosslinking, and Immunoprecipitation** - Confluent monolayers of control CHOK1 cells, and CHOK1 cells transfected with Noggin, were labeled overnight with 100 µCi/ml $^{35}$S-Na$_2$SO$_4$ (ICN) in S-MEM media containing dialyzed FCS (Gibco). Cells were washed three times with PBS, DTSSP (Pierce) was added to a final concentration of 5 mM, and incubated for 30 min at 4°C (24). Monolayers were washed three times with TBS to quench the crosslinking reaction, followed by three additional washes with PBS, and then subsequently extracted and immunoprecipitated as described above. After the final PBS wash, the beads were resuspended in 100 µl of 20 mM Tris pH 7.5 containing 5 mM CaCl$_2$ and then incubated for 180 min at 37°C plus or minus two additions of Heparitinase I (Sigma) of 2 mU/ml spaced 90 min apart. Following two final washes with PBS, the immunoprecipitates were eluted with 2x SDS loading buffer (BioRad) containing β-mercaptoethanol, boiled, centrifuged and analyzed on a 4-20% gradient SDS-PAGE gel (BioRad).

**Heparin binding affinity** - A column containing 1 ml of heparin-Sepharose was prepared and attached to an AKTA FPLC unit (Amersham-Pharmacia) and run at a flow rate of 1 ml/min. The resin was equilibrated in 20 mM Tris-HCl, pH 7.4 and then 10 µg of purified recombinant Noggin in the same buffer was loaded and the column washed for 10 column volumes. Bound
protein was eluted in the same buffer using a linear gradient up to 2 M NaCl over 20 column volumes. For comparisons of hNog and hNogΔB2 binding to heparin, 160 microliters of heparin-Sepharose (Sigma) was packed into a mobicol column (Mobitec). Following equilibration with 20 mM Tris-HCL, pH 7.5, supernatants containing 250 ng of hNog or hNogΔB2 were applied to each column. Columns were washed with 10 ml of equilibration buffer and subsequently eluted sequentially with two column volumes each of 20 mM Tris pH 7.5 with increasing amounts of salt. Eluted samples were separated by SDS-PAGE electrophoresis and Noggin detected by Western as described above.

**Immunofluorescence**- 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) for 30 min at 37°C, then washed twice with warm medium, and incubated with secondary antibodies containing 0.01 mg/ml Hoechst for 30 min at 37°C. After washing twice with PBS, the cells were fixed with 4% paraformaldehyde in PBS for 10 min., washed twice with PBS followed by a water rinse, and then mounted in ProLong Antifade (Molecular Probes).

Immunofluorescence microscopy was performed with an Olympus Fluoview 500 configured with Krypton and UV with the appropriate wavelength filters (568 and 351 nm) for CY3 and Hoechst excitation. Confocal images were assembled as montages using Adobe Photoshop.

**125I-BMP-4 Binding, Affinity Cross-linking, and Analysis of Complexes**- BMP4 (R and D Systems) was labeled with 125I using Iodogen tubes (Pierce). Confluent monolayers of cells
were washed 3 times with PBS at 4°C. 100 ng/ml of $^{125}$I-BMP4 was added and following incubation for 90 min at 4°C with gentle shaking, cells were washed 3 times with PBS. DTSSP (Pierce) at 5mM in PBS was added to the monolayer and incubated for 30 min at 4°C. The crosslinking reaction was halted by washing 3 times with TBS and then twice with PBS. Cell layers were extracted and immunoprecipitated as described above.

**Osteogenic Differentiation Assay** - C2C12 myoblasts were maintained in DMEM/F12 supplemented with 10% FCS. Cells were plated in 24 well plates to be 70% confluent after incubating at 37°C overnight. Dilutions of supernatants containing Noggin or ΔB2 Noggin were mixed with BMP4 and allowed to incubate at room temperature for 30 min, at which point they were added to the C2C12 cells and incubation extended overnight at 37°C. Following cell lysis, alkaline phosphatase activity was measured using a colorimetric assay (Sigma).

**Results**

**Noggin binds to heparin-Sepharose** - To establish whether Noggin is a heparin binding protein, we evaluated the ability of purified recombinant Noggin to bind to heparin-Sepharose *in vitro*. Recombinant Noggin was purified to homogeneity as assessed by detection of a single peak on RP-HPLC (C4 Vyda Column, 20%-60% CH$_3$CN/H$_2$O over 40 min), and the identity of this product as Noggin confirmed by N-terminal amino acid sequence analysis. Purified Noggin (10 µg) was applied to a heparin-Sepharose affinity column and eluted with a linear salt gradient from 0 to 2 M NaCl. Aliquots of eluted fractions were assessed by 10-20% SDS-PAGE and
Western blotting with a rat anti-human monoclonal antibody (RP57-16). As shown in Figure 1 A, Noggin eluted from the column as a sharp peak only at high salt concentration. Indeed, greater than 1 M NaCl was found to be required to effect elution of Noggin from heparin-Sepharose.

Close inspection of the primary sequence of Noggin reveals a cationic stretch of amino acids between residues 130-147. When plotted as a helical wheel (Fig. 1 B), these cationic residues line up predominantly on one face of the helix, suggesting that they may form a portion of a heparin-binding domain. Indeed, a Noggin mutant engineered to exhibit reduced binding to heparin by deleting amino acids 133 to 144, has a reduced affinity for heparin and displays reduced clearance upon administration to adult mice (23). This is illustrated in Figure 1 C where in contrast to wild type Noggin, which again elutes predominantly at 1 M NaCl, the hNogΔB2 mutant elutes from heparin-Sepharose at between 0.2 and 0.4 M NaCl. This suggests that this sequence contains residues important for the binding of Noggin to heparin.

The salt requirement for elution of Noggin from heparin-Sepharose is similar to that which is required for elution of a number of proteins known to have physiologically relevant interactions with heparan sulfate proteoglycans in vivo. This includes, for example, some members of the fibroblast growth factor gene family known to require binding to heparan sulfate for function (25,26). This suggested to us that Noggin may bind to heparan sulfate proteoglycans in vivo and that, in particular, cell surface heparan sulfate proteoglycans might serve to bind and localize Noggin to the plasma membrane.

**Noggin localizes only to the surface of cells expressing heparan sulfate proteoglycans** - To test
the hypothesis that cell surface heparan sulfate proteoglycans bind Noggin \textit{in vivo}, we stably expressed human Noggin in normal cultured cell lines, as well as in cell lines bearing known mutations in the heparan sulfate biosynthetic pathway. These included CHO cell lines deficient in both heparan sulfate and chondroitin sulfate biosynthesis (PGSA-745) or heparan sulfate biosynthesis alone (PGSD-677), as well as the parental cell line (CHOK1) which displays normal heparan sulfate biosynthesis (27-29). These three cell lines were cotransfected with a human \textit{noggin} expression plasmid (pSRα.hNG) as well as the selectable marker plasmid pSVZeo to allow for the positive selection of stable cell lines.

Following stable selection in Zeocin, individual transfected lines were subcloned and SDS-PAGE and Western blotting with a monoclonal antibody specific to Noggin was used to screen whole cell extracts and condition media from Zeocin resistant subclones. Individual clones were selected for further study that demonstrated similar levels of Noggin expression in the cell layer and conditioned media (Fig. 2 A).

We evaluated whether the presence or absence of heparan sulfate had any effect on the cellular distribution of Noggin in transfected CHO cells. Specifically, cell surface Noggin was detected by immunofluorescence of non-permeablized cells with Noggin specific rat monoclonal antibodies and CY3 secondary antibodies. As shown in Figure 2, CHOK1 cells stably expressing human Noggin display readily detectable levels of Noggin at the cell surface (Fig. 2 C), while the untransfected parental cell line does not (Fig. 2 B). In contrast, neither PGSA-745 (Fig. 2 D) nor PGSD-677 (Fig. 2 E) cells expressing similar levels of human Noggin express any detectable levels at the cell surface. Cells permeabilized prior to immunofluorescent staining reveal similar levels of vesicular intracellular staining of Noggin, presumably in the secretory
and/or endocytic pathways (data not shown).

To confirm that the cell surface staining results were not somehow merely due to an inaccessibility of the Noggin epitope when bound at the surface of heparan sulfate deficient cells, we compared the staining pattern of these cells using several additional antibodies. These included two additional monoclonal antibodies (RP57-06 and RP57-21), at least one of which is known to bind to a different region of Noggin (Regeneron, unpublished data), as well as a polyclonal antiserum against Xenopus Noggin that cross-reacted with human Noggin. All antibodies produced identical staining results (data not shown). Furthermore, parental CHOK1 cells expressing similar levels of hNogΔB2 when stained with anti-Noggin antibody also reveals no detectable protein at the cell surface (data not shown). Taken together with the observations from the PGSA-745 and PGSD-677 cells, which lack the ability to synthesize heparan sulfate and also fail to bind wild-type Noggin at their cell surface, these results suggest that Noggin binding to the cell surface is a heparin-dependent phenomenon.

Cell surface bound Noggin is displaced specifically by heparin- To confirm that cell surface binding of Noggin is heparin-dependent, we determined whether Noggin could be displaced from the surface of CHO cells by soluble glycosaminoglycans. CHOK1 cells, stably expressing human Noggin, were pulse labeled with Translabel- 35S for 30 min and then chased in unlabeled media in the presence or absence of glycosaminoglycans. Labeled Noggin was detected in the total cell layer and media at each time point by immunoprecipitation and SDS-PAGE followed by autoradiography. As seen in Figure 3 A, newly synthesized Noggin has a cellular half-life of approximately 3.6 h and is released into the medium (Fig. 3 E). However, the total labeled
Noggin recovered from the medium is not equivalent with that which is synthesized in the initial 30 min pulse, suggesting some proportion of cell surface Noggin is likely to be internalized by the cell and degraded. In contrast, cells that are pulse labeled and then chased in the presence of soluble heparin show almost immediate release of Noggin from the cell surface (Fig. 3 B). Concomitant with this disappearance of cell surface Noggin is the rapid, and apparently stable, accumulation of Noggin in the cell medium (Fig. 3 F), indicating that heparin is displacing Noggin from the cell surface. The total labeled Noggin recovered from the medium is quantitatively similar to that which is initially synthesized by the cells during the pulse. This suggests that Noggin, once displaced from the surface by heparin, is protected against subsequent internalization and degradation, and suggests that Noggin may normally be endocytosed in a heparin-dependent manner. In support of this conclusion is the finding that the hNogΔB2 mutant of Noggin, with reduced heparin binding, shows rapid release from CHOK1 cells (Fig. 3 D) and accumulates stably in the media (Fig. 3 H) like heparin treated cells expressing wild type Noggin. Although a slightly reduced molecular weight form of the hNogΔB2 mutant of Noggin is evident in the media, suggesting perhaps increased proteolytic processing of this mutant, this observation has been inconsistently observed and we are therefore uncertain of its significance.

To confirm that displacement of Noggin from the cell surface is not simply a non-specific effect of the addition of a highly negatively charged polysaccharide, we evaluated the ability of an equal molar concentration of chondroitin sulfate to displace cell surface Noggin. As shown in Figures 3 C and 3 G, addition of chondroitin sulfate had no effect on the turnover or cellular localization of Noggin.
**Noggin directly binds to cell surface proteoglycans** - Noggin remains localized only on the surface of those cells capable of heparan sulfate biosynthesis. Since this binding can be displaced by heparin but not by chondroitin sulfate, this suggests that the primary site of interaction of Noggin with the plasma membrane is through a sulfate containing proteoglycan. We therefore wanted to directly assay for a direct physical association between Noggin and a heparan sulfate containing proteoglycan at the cell surface.

To evaluate this, CHOK1 cells expressing Noggin were metabolically labeled with $^{35}$S inorganic sulfate to label proteoglycans. Following crosslinking with a small chain reducible agent to crosslink interacting proteins, we isolated complexes by immunoprecipitation with anti-Noggin antibodies and evaluated them by reduced SDS-PAGE and autoradiography. As shown in Figure 4 (lane 2), a sulfate labeled protein that migrates with the electrophoretic characteristics of a proteoglycan was isolated in association with Noggin. No similar product was immunoprecipitated from control cell lines not expressing Noggin (Fig. 4, lane 1). Furthermore, the immunoprecipitate is sensitive to enzymatic cleavage with Heparitinase I (lane 3) confirming the identity of this material as a heparan sulfate proteoglycan. These results support the conclusion that Noggin is bound to heparan sulfate proteoglycans at the cell surface.

**Noggin bound to the plasma membrane by heparan sulfate binds BMP4 at the cell surface** - Noggin is well characterized as a BMP antagonist, which binds BMP4 with high affinity (2). Since the binding of heparan sulfate to the heparin binding domain of Noggin could in principle block the ability of Noggin to bind BMP4, we wished to establish that the cell surface Noggin bound to heparan sulfate remained functional to bind BMP4. Noggin expressing and non-expressing
CHOK1 and PGSA-745 cells were incubated at 4°C with 125I-labeled BMP4 to allow binding to the cell surface, but to prevent endocytosis. Following crosslinking with a reducible short chain agent to crosslink interacting proteins, complexes immunoprecipitated with anti-Noggin antibodies were evaluated by SDS-PAGE, with and without reduction, followed by autoradiography. As expected, radiolabeled BMP4 is not recovered from cells that do not express Noggin (Fig. 5 lanes 1, 3, 5, and 7). Furthermore, no BMP4 is immunoprecipitated with anti-Noggin antibodies from those cells expressing Noggin, but deficient for heparan sulfate biosynthesis and therefore lacking in membrane bound Noggin (Fig. 5, lanes 4 and 8). In contrast, cells with Noggin bound to heparan sulfate at the cell surface demonstrate the inclusion of BMP4 into a high molecular weight complex (Fig. 5, lane 2). The high molecular weight of this complex is predicted based on its inclusion of BMP4, Noggin, and presumably the high molecular weight heparan sulfate proteoglycan that binds Noggin as shown in Figure 4. Following reduction, as predicted, one detects a single low molecular weight band of the expected size for labeled BMP4 (Fig. 5, lane 6). Binding of BMP4 to Noggin produced by these cells is specific because binding is both saturable and can be inhibited by cold BMP4 (data not shown). These results demonstrates that Noggin can be localized to the plasma membrane of cells by heparan sulfate proteoglycans in an active form that binds BMP4.

The heparin binding domain of Noggin is independent of its function as a BMP antagonist- Noggin deleted for the heparin binding domain is not detected on the plasma membrane of transfected cells by immunofluorescence (as shown above). Consistent with these results, the deletion mutant is rapidly released from the cell surface during pulse chase experiments, and no
cell surface bound $^{125}$I-labeled BMP4 can be immunoprecipitated with anti-Noggin antibodies from these same cells (data not shown). Despite the altered cellular distribution of this mutant, it remains functional as a BMP antagonist. We compared the binding affinity of both Noggin and the hNogB2 mutant for BMP4 by equilibrium binding analysis and determined identical Kd values of 30 pM (data not shown), which are in agreement with previously published values for Noggin (2). Furthermore, this mutant shows similar function to wild-type Noggin in bioassays of Noggin activity as a BMP antagonist. For example, the mutant has similar activity to wild-type Noggin in the ability to inhibit membranous ossification of bone in rats (30), a process presumed to be mediated by the induction of endogenous BMPs in response to bone fracture.

As a further direct comparison of the function of these two proteins, we compared their ability to antagonize BMP activity in a simple cell based assay. C2C12 myoblasts are muscle progenitor cells that differentiate into myotubes upon withdrawal of mitogens. When treated with BMPs, however, this cell line undergoes transdifferentiation along the osteoid lineage as assessed by the induction of alkaline phosphatase (31). As shown in Figure 6 A, alkaline phosphatase induction is dose responsive in these cells over a range of BMP4 concentrations from 0.5 to 5 nM. When cells are treated with 1 nM BMP4 in the presence of either Noggin or hNogB2, these two proteins both inhibited induction of alkaline phosphatase, with hNogB2 showing slightly reduced but comparable activity to that of Noggin (Fig. 6 B).

**Discussion**

Regulation of cell fate is essential for proper patterning events in developing organisms.

Control of this process is dependent on a combination of both short and long range acting
signaling molecules. It has been hypothesized that the diffusion of secreted BMP antagonist from organizer regions might establish gradients of BMP antagonism, resulting in turn in inverse activity gradients of BMP (Fig. 7 A) (12,14,18,19). Local destruction of these antagonists by specific extracellular proteases is one potential mechanism for further regulating the formation of these gradients (8,9,32). In contrast, little is known about the potential binding sites at the cell surface or within the extracellular matrix that might regulate the formation of these gradients by controlling the diffusion of these antagonists. We report here that the BMP antagonist Noggin binds strongly to heparan sulfate proteoglycans, and that this interaction serves to localize secreted Noggin to the plasma membrane in vitro where it remains functional to bind BMP.

Heparan sulfate containing proteoglycans are found ubiquitously in vivo, both on the surface of all adherent cells as well as within the extracellular matrix surrounding those same cells (20). Proteins bearing this specific post-translational modification have essential roles in patterning events, as emphasized by the identification of numerous vertebrate and invertebrate mutants in heparan sulfate biosynthesis, which have dramatic defects in morphogenesis (33). Although the pathways affected in these mutants are in many cases known, in most cases the mechanisms by which they are effectuated are still poorly understood.

In the case of BMP, members of the glypican gene family of glycoprophosphatidylinositol(GPI)-linked heparan sulfate proteoglycans appear to play an important role in the control of cellular responsiveness to this morphogen. Mutations in the Drosophila glypican dally cause defects in post-embryonic morphogenesis associated with an alteration in cellular responsiveness to the Drosophila ortholog of vertebrate BMP, decapentaplegic (Dpp) (21). In vertebrates, we have recently shown that loss of function
mutation in \textit{glypican-3}, which causes Simpson Golabi Behmel dysmorphia syndrome in humans and mice, results in polydactyly and other skeletal defects in association with a loss of cellular responsiveness to BMP4 (22). In neither case is it clear by what mechanism these heparan sulfate proteoglycans are serving to effect cellular responsiveness to BMP. Unlike the situation with fibroblast growth factors, there does not appear to be any essential requirement for heparan sulfate as a coreceptor for BMP.

Our results described here show that heparan sulfate proteoglycans can regulate the cellular distribution of a BMP antagonist, Noggin, and therefore offer one potential mechanism through which heparan sulfate proteoglycans could regulate cellular responsiveness to BMPs \textit{in vivo}. We have shown that Noggin binds strongly to heparin \textit{in vitro}, and to heparan sulfate on the surface of cultured cells. This suggests that the diffusion of Noggin \textit{in vivo} is likely to be affected by the presence or absence of heparan sulfate proteoglycans with high affinity for Noggin at and near the source of its secretion. Although heparan sulfate itself is ubiquitous, its structure is extremely heterogeneous. There is evidence that the structure of heparan sulfate chains found \textit{in vivo} may be both spatially and temporally regulated to impart tissue specific preference for certain ligands over others (34,35). Rather than diffusing freely from a local site of production to form simple gradients of BMP antagonism, as indicated in Figure 7 A, we would propose that heparan sulfate sequences with high affinity for Noggin \textit{in vivo} are likely to control Noggin diffusion. Therefore, heparan sulfate proteoglycans in a field of developing tissue may play an essential role in determining the shape of the BMP activity gradient formed as suggested in Figure 7 B. These could include cell surface heparan sulfate proteoglycans of either the glypican or syndecan gene families, as well as pericellular heparan sulfate proteoglycans.
such as perlecan, agrin or type XVIII collagen, all of which are found abundantly \textit{in vivo}. The relative activity of each of these heparan sulfate proteoglycans in this function would be dependent on their individual concentrations in the local environment, as well as their individual affinities for Noggin.

Important to the model is our discovery that the binding of Noggin to heparan sulfate is independent of its activity as a BMP antagonist, inasmuch as Noggin bound to cell surface heparan sulfate retains its ability to bind BMP. This is further supported by the crystal structure of Noggin bound to BMP7 (Jay Groppe, Jason Greenwald, Aris N. Economides, Markus Affolter, and Senyon Choe, in preparation) and the findings that Noggin variants with mutations in heparin binding show no significant difference in an osteogenic differentiation assay in culture (this study) or a membranous ossification assay \textit{in vivo} (30). This does not necessarily mean, however, that the role of heparan sulfate proteoglycans \textit{in vivo} is merely as a passive sink for Noggin and bound BMP. Our pulse chase experiments indicate that only a portion of newly synthesized Noggin is actually released from the plasma membrane of cultured cells and accumulates in the media. The remainder is presumably internalized and degraded. When these cells are treated with heparin, all of the Noggin is rapidly displaced from the plasma membrane and accumulates stably within the media. Furthermore, the hNogΔB2 mutant of Noggin, which displays reduced heparin binding, behaves similarly without any addition of heparin. This implies that heparan sulfate proteoglycans could play an essential role in the uptake and degradation of Noggin and its bound ligands as well. Heparan sulfate proteoglycans are known to be important in the uptake and degradation of a wide range of extracellular proteins (20). In particular, a similar role has been proposed previously for heparan sulfate in the turnover of
another BMP antagonist, Follistatin, and its bound ligands (36,37).

Active uptake of Noggin through cell surface heparan sulfate has other potential implications for gradient formation that remains to be tested. Recent studies have suggested that Wingless in *Drosophila* imaginal disc tissue may disperse over large distances without release from the plasma membrane. This process can apparently be mediated by the direct transfer between cells of specialized vesicles, called argosomes, rich in GPI-anchored proteins (38). Uptake and localization of Wingless into argosomes is dependent on cell surface heparan sulfate, suggesting that the GPI-linked *Drosophila* glypicans *dally* and *dally-like* might serve to sort Wingless into these structures (38). This would offer a molecular explanation for why mutations in these glypicans disrupt Wingless signaling *in vivo* (39-41). By analogy, a similar process could disperse Noggin bound to the plasma membrane through glypicans, providing another mechanism for the control of BMP activity gradient formation *in vivo* by heparan sulfate proteoglycans.

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References


39. Tsuda, M., Kamimura, K., Nakato, H., Archer, M., Staatz, W., Fox, B., Humphrey, M.,


Figure Legends

Fig. 1 Noggin is a heparin-binding protein. (A) 10 µg of purified recombinant Noggin was loaded onto a heparin-Sepharose column, washed, and then eluted with a linear gradient of salt up to 2 M NaCl. Eluted fractions were evaluated by Western blotting to identify the peak of Noggin elution identified by the arrow. Note small amounts of high molecular weight material, likely representing aggregates of improperly folded Noggin, elutes at low salt concentration. (B) Helical wheel projection of Noggin amino acids 130-147 showing alignment of basic residues primarily on a single face of the alpha helix. (C) Comparison of NaCl elution profiles of Noggin (hNog) and ΔB2 Noggin (hNogΔB2) from heparin-Sepharose showing weaker binding of ΔB2 Noggin.

Fig. 2 Noggin localizes only to the surface of cells expressing heparan sulfate. Cell layer extracts and conditioned media from stable cell clones transfected with a noggin expression plasmid were evaluated by Western blotting to identify lines showing similar levels of expression (A). Composite phase and immunofluorescence confocal images of unpermeabilized CHO cells stained with an anti-Noggin monoclonal antibody (RP57-16), detected with a species specific CY3 secondary antibody, and nuclear counter stained with Hoechst. CHOK1 cells stably expressing human Noggin display detectable levels of Noggin on the cell surface in a punctate distribution pattern (C), while untransfected cells do not (B). By contrast, mutant CHO cell lines lacking heparan sulfate, but expressing similar levels of Noggin, have no detectable cell surface Noggin. PGSA-745 cells (D) lack both heparan and chondroitin sulfate while PGSD-677 cells (E) lack only heparan sulfate.

Fig. 3 Heparin selectively promotes stable release of cell surface bound Noggin into the media.
CHOK1 cells stably expressing Noggin were pulse labeled with Translabel-35S in methionine- and cysteine-free media, and analyzed by immunoprecipitation with anti-Noggin antibodies followed by SDS-PAGE. The majority of newly synthesized Noggin remains associated with the cell layer (A), with the remaining fraction secreted into the culture medium (E). Treatment of these same cells with 1 µg/ml of heparin during the pulse chase resulted in rapid release of all labeled Noggin from the cell layer (B) and the stable accumulation of this Noggin within the culture medium (F). In contrast, treatment of these cells with 1 µg/ml chondroitin sulfate had no effect on the fate of synthesized Noggin (C and G), suggesting that the effect of heparin is specific and represents competition for heparan sulfate binding sites on the cell surface. Consistent with these observations, the hNogΔB2 deletion mutant of Noggin, with reduced binding to heparin, is rapidly released from CHOK1 cells (D) without heparin treatment and shows stable accumulation within the medium (H).

Fig. 4 Noggin binds to 35S-labeled cell surface proteoglycans. CHOK1 cells stably expressing Noggin (lanes 2 and 3) or a control vector (lane 1) were metabolically labeled for 24 hours in culture media containing 50 µCi/ml 35S-inorganic sulfate to label sulfated proteoglycans. Following treatment with DTSSP, cell extracts were immunoprecipitated with anti-Noggin antibodies and evaluated by SDS-PAGE and autoradiography. Antibodies to Noggin immunoprecipitated a sulfate labeled proteoglycan from cells expressing Noggin (lane 2) but not from control cells (lane 1). The immunoprecipitated material is sensitive to digestion with Heparitinase I (lane 3), confirming it to be a heparan sulfate proteoglycan.

Fig. 5 Noggin bound to heparan sulfate binds BMP4 at the cell surface. Noggin expressing
(Noggin +) and nonexpressing (Noggin -) CHO cells that either express heparan and chondroitin
sulfate glycosaminoglycans (GAG +) or do not (GAG -), were incubated at 4°C with \(^{125}\text{I-}
\)labeled BMP4, treated with DTSSP and immunoprecipitated with anti-Noggin antibodies.
Radiolabeled BMP4 is not recovered from cells that do not express Noggin (lanes 1, 3, 5, and 7),
nor is it recovered from those Noggin expressing cells that do not synthesize heparan sulfate
(lanes 4 and 8). In contrast, Noggin and heparan sulfate expressing cells include BMP4 into a
high molecular weight complex (lane 2), which releases BMP4 following reduction (lane 6).

Fig. 6 Noggin and NogginB2 deleted for the heparin-binding domain show comparable potency
as a BMP4 antagonist. (A) C2C12 myoblasts treated with increasing concentrations of BMP4 for
24 hours at 37°C and then assayed for alkaline phosphatase activity as a marker of osteogenic
transdifferentiation. (B) C2C12 cells treated with 1 nM BMP4 in the presence of decreasing
concentrations of either Noggin (White bars) or NogB2 (Gray bars). NogB2, which is deleted for
the heparin-binding domain, shows slightly reduced but comparable activity to Noggin as an
antagonist of BMP4 transdifferentiation of C2C12 cells.

Fig. 7 Model for the potential role of heparan sulfate in shaping BMP activity gradients in vivo.
(A) Model for the role of Noggin in the formation of BMP activity gradients in vivo. Noggin
secreted from a focal source diffuses outward, forming a graded distribution of BMP antagonism.
This results, in turn, in an inverse BMP activity gradient. (B) Expression of heparan sulfate
proteoglycans, with high affinity for Noggin, along the path of Noggin diffusion could restrict or
modify the diffusion of Noggin and thus modifying the pattern of the resulting BMP activity
gradient. Yellow- Bmp gene expression pattern. Blue- Graded diffusion of Noggin from site of
expression. Green- Resulting BMP activity gradient. Red- Expression of heparan sulfate
proteoglycans that bind Noggin.
Crosslinking: α-noggin IP

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Heparan sulfate proteoglycans retain noggin at the cell surface: A potential mechanism for shaping BMP gradients
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