Cell-surface Heparan Sulfate Proteoglycans Potentiate Chordin Antagonism of Bone Morphogenetic Protein Signaling and Are Necessary for Cellular Uptake of Chordin*

Received for publication, July 19, 2004, and in revised form, September 13, 2004
Published, JBC Papers in Press, September 20, 2004, DOI 10.1074/jbc.M408129200

Reema Jasuja‡§, Benjamin L. Allen¶¶, William N. Pappano§*†, Alan C. Rapraeger‡‡‡‡‡‡, and Daniel S. Greenspan‡***‡‡‡‡‡

From the Programs in ‡Molecular and Cellular Pharmacology and ¶¶Cellular and Molecular Biology and the Departments of *Biomolecular Chemistry and ‡‡Pathology and Laboratory Medicine, University of Wisconsin, Madison, Wisconsin 53706

Transforming growth factor-β (TGF-β)-like bone morphogenetic proteins (BMPs) were first isolated from osteogenic ex-
HSPGs Regulate Chordin Function, Uptake, and Diffusion

EXPERIMENTAL PROCEDURES

Production of Recombinant Proteins—FLAG-tagged mouse Chordin, FLAG-tagged Chordin fragments, and protein C-tagged mouse TSG were expressed and purified as described previously (19, 25). Concentrations of BMP-4 (R&D Systems), FLAG-tagged mouse Chordin, FLAG-tagged Chordin fragments, and protein C-tagged mouse TSG were calculated by comparing the intensities of Coomassie Blue-stained bands with protein standards of known concentrations.

Heparin-Sepharose Chromatography—A heparin-Sepharose CL-6B slurry (Amersham Biosciences) was pelleted and resuspended in a double volume of phosphate-buffered saline (PBS) containing 1 mg/ml bovine serum albumin (BSA), and 100 μl were added to separate equimolar amounts of BMP-4, Chordin, or protein C-tagged mouse TSG or to equimolar combinations of these proteins that had been preincubated together for 30 min at 37 °C. Heparin-Sepharose/protein mixtures were incubated for 2 h at 4 °C and then loaded onto a 0.8 × 4-cm column, and the flow-through was collected. The column was washed with 500 μl of PBS containing 1 mg/ml BSA, and stepwise elution was performed using 75-μl aliquots of PBS containing increasing concentrations of NaCl. Elution was followed by addition of 4× SDS-PAGE sample buffer with 5% β-mercaptoethanol to fractions and electrophoresis on 4–15% acrylamide gradient gels (Bio-Rad). Immunoblotting by electrotransfer to polyvinylidene difluoride membranes, incubations of blots with antibodies, and washes were performed as described previously (41). Rabbit polyclonal antibody raised against peptide EPPALPISEIKEPVRGLVA (20), corresponding to residues 30–48 at the N terminus of the published mouse Chordin sequence (42); anti-BMP-4 monoclonal antibody (R&D Systems); peroxidase-conjugated anti-protein C monoclonal antibody (Roche Applied Science), for detection of protein C epitope-tagged mouse TSG; biotinylated anti-FLAG monoclonal antibody (Sigma); and streptavidin-horseradish peroxidase conjugate and secondary antibodies (Amersham Biosciences) were all used at 1:5000.

Cell Binding Assays and Immunofluorescence—Raji cells stably transfected with syndecan-1 (Raji-S1) (43), Raji cells similarly transfected with syndecan-4 (Raji-S4), and parental Raji cells cultured as described previously (44), and 101/2 cells cultured in Dulbecco’s modified Eagle’s medium (DMEM) and 10% fetal bovine serum (FBS) (HyClone Laboratories) were fixed by incubation with 4% paraformaldehyde in PBS for 1 h. Fixed cells were then incubated with or without 60 nm Chordin in RPMI 1640 medium and 10% FBS for 1 h at room temperature. Three 5-min washes with PBS were followed by incubation with anti-Chordin antibody (described above) at 1:200, with rat anti-syndecan-1 monoclonal antibody 281.2 at a concentration of 1 μg/ml, or with rabbit anti-syndecan-4 polyclonal antibody (see below) at 1:1 h. This was followed by three 5-min washes with PBS and then incubation with Alexa 546-conjugated goat anti-rabbit antibody or Alexa 488-conjugated donkey anti-rat antibody (Molecular Probes, Inc.) at 1:1000 for 30 min, followed by a final three 5-min washes with PBS. Rabbit anti-syndecan-4 polyclonal antibody was made using a syndecan-4 exodomain-glutathione S-transferase fusion protein. The antibody was puriﬁed by glutathione S-transferase affinity chromatography. Cell-surface protein cores include the syndecans (27), CD44 (V3) (28, 29), and betaglycan (30), all of which have transmembrane domains; and the glypicans, which bind plasma membranes via glycosylphosphatidylinositol linkages (31). In both interstitial and basement membrane extracellular matrix, perlecans are the primary core protein for HS attachment (32, 33).

HSPGs are implicated in diverse functions that include cell binding to the extracellular matrix and to various growth factors (32). Secreted growth factors that interact with HSPGs include, but are not limited to, fibroblast growth factors (FGFs), the epidermal growth factor and platelet-derived growth factor (34). HSPGs have been shown to interact with various developmentally important ligands, including Wnt family members (34) and TGF-β-like BMPs (35, 36). With respect to the latter, the Drosophila glypican dally is required for normal DPP signaling (36), whereas HS may be capable of modulating BMP-2 activities in cell culture and embryonic chick limb bud assays (35, 37, 38). Recently, it has been shown that Noggin binds to HSPGs (39).

Here, we demonstrate that Chordin binds heparin-Sepharose with elution profiles similar to those of proteins, such as FGFs, that are known to functionally interact with HSPGs in tissues. We show that, surprisingly, mammalian TSG did not bind heparin, in contrast to Drosophila TSG (40), but that it would bind heparin if complexed to Chordin and/or TSG. We show that Chordin bound embryonic tissues in a manner that was dependent upon its interactions with tissue HSPGs and that Chordin bound cell-surface HSPGs and specifically cell-surface syndecans, but that it did not detectably bind basement membranes containing the major HSPG perlecans. Importantly, the results presented herein indicate that cell-surface HSPGs strongly potentiate Chordin antagonism of BMP signaling, effect the retention of Chordin at cell surfaces, and are necessary for the cellular uptake of Chordin. Implications of the various data for the modulation of BMP signaling and the formation of BMP signaling gradients are discussed.
RESULTS

Chordin and BMP-4, but Not Mouse TSG, Bind Heparin In Vitro—To gain insight into the potential of Chordin to bind HS in vivo, purified recombinant murine Chordin was incubated with heparin-Sepharose, and the mixture was placed in a column and eluted with a step gradient of 0.15–2.0 M NaCl. As shown in Fig. 1A, Chordin bound heparin, with the majority of bound Chordin eluting at 0.55–1.60 M NaCl and some requiring elution with 2.0 M NaCl or boiling in SDS. It has previously been shown that TGF-β-like BMPs (e.g. BMP-2) (35) bind heparin with high affinity. Here, BMP-4 was shown to bind heparin with an elution profile similar to that of Chordin (Fig. 1A). Murine TSG did not bind heparin under these conditions, as the vast majority was found in the flow-through and wash (Fig. 1A). This result was surprising, as Drosophila TSG has been reported to be a heparin-binding protein (40).

Chordin binds BMP-4 and murine TSG and can bind the two proteins simultaneously in a ternary complex (24–26). To determine whether binding to mouse TSG might decrease the affinity of Chordin and/or BMP-4 for heparin or whether binding of TSG to the other two proteins might increase its retention on heparin, the three proteins were preincubated together prior to incubation with heparin-Sepharose. As shown in Fig. 1B, in the presence of Chordin and BMP-4, some portion of the input murine TSG was retained on heparin and was eluted at fairly high concentrations of NaCl, similar to those at which Chordin and BMP-4 were eluted. As only some portion of the input TSG was expected to bind Chordin and/or BMP-4 during preincubation, it seems likely that the portion retained on heparin-Sepharose represents that portion of mouse TSG that was bound to Chordin and/or BMP-4. Mouse TSG preincubated separately with either Chordin or BMP-4 was retained on heparin-Sepharose in amounts and with affinities similar to those of TSG preincubated with the two proteins together (data not shown). Thus, binding to Chordin and/or BMP-4 is necessary/sufficient for murine TSG binding to heparin.

The extracellular BMP antagonist Noggin binds heparin predominantly via a short segment of sequence that includes a stretch of basic amino acid residues (39). Similarly, BMP-2 binds heparin predominantly via a stretch of basic amino acids near its N terminus (35), and the highly similar protein BMP-4 most probably binds heparin via an N-terminal region even more basic than that of BMP-2. Full-length Chordin contains four cysteine-rich domains that are thought to be involved in BMP binding (Fig. 2A)/(2)/(50). To determine whether Chordin might also bind heparin via a site localized to a relatively small portion of the protein, recombinant versions of each of the three cysteine-rich domain (CR)-containing fragments that are generated via cleavage of Chordin by BMP-1/Tolloid-like metalloproteinases (19) were prepared as described (25) and incubated separately with heparin-Sepharose. As shown in Fig. 2B, the N-terminal CR1-containing fragment, the middle CR2/CR3-containing fragment, and the C-terminal CR4-containing fragment all bound heparin, with elution occurring at relatively high concentrations of NaCl. Some of the CR1-containing fragment and large amounts of the CR4-containing fragment were also found in the flow-through and wash, perhaps reflecting improper folding by some fraction of these recombinant fragments. Nevertheless, binding by all three fragments suggests that binding of Chordin to heparin is via more than one site, consistent with the relatively even distribution of basic residues in the Chordin sequence (42, 50).

Chordin Binds Tissues in an HSPG-dependent Manner—Next, we directly tested the ability of Chordin to bind endogenous HSPGs in embryonic tissues. It has previously been shown that proteins that bind HSPGs in vivo can, when added exogenously to tissue sections, bind in a manner dependent upon interactions with endogenous HSPGs and that different endogenous HSPGs show selectivity for specific ligands (44,
thought to be required for interaction with BMPs. The full-length Chordin, including four cysteine-rich domains, which are thought to be required for interaction with BMPs. The arrows indicate defined sites of cleavage by BMP-1/Tolloid-like proteases. B, recombinant, FLAG-tagged, CR-containing cleavage products were incubated with heparin-Sepharose, followed by elution at increasing NaCl concentrations. SDS-PAGE was performed on the elution products, followed by Western blot analysis with monoclonal anti-FLAG antibody.

To determine whether exogenous Chordin might selectively bind tissues in an HSPG-dependent fashion, recombinant Chordin was incubated on frozen sections from 17-day postcoitus (dpc) mouse embryos. In such assays, Chordin was found to bind tissues in a manner dependent upon interactions with endogenous HS since binding was blocked by pretreatment of tissues with heparin lyases (heparinas I and III) (Figs. 3–5). In contrast, pretreatment of tissue sections with chondroitin ABC lyase, which removes the side chains of endogenous chondroitin sulfate proteoglycans, had no effect on Chordin binding (data not shown).

In skin, a relatively weak HS-dependent signal was observed for Chordin bound to epidermis and hair follicles (Fig. 3A). However, mast cells, which served as internal controls for Chordin binding in skin since these cells produce heparin and contain high levels of heparin stored in α-granules, showed relatively high signal levels for Chordin binding. This latter result is consistent with the probability that binding of Chordin to tissues is via the same types of interactions by which it binds heparin-Sepharose in vitro. Interestingly, Chordin did not detectably bind the HSPG-rich basement membrane at the dermal-epidermal junction.

In the thorax, the signal for Chordin binding was at particularly high levels in developing ribs (Fig. 3B) and lung (Fig. 4A). Interestingly, in situ hybridization has previously shown Chordin to be expressed at high levels in both of these tissues (51). In ribs and lung, the tissue distribution of HS was demonstrated using mAb 3G10, which binds to residual HS stubs that remain on endogenous HSPG core proteins after heparin lyase treatment (46). As shown in Figs. 3B and 4A, the use of mAb 3G10 showed both ribs and lung to be abundant in endogenous HS. Moreover, binding of Chordin to serial sections showed the signal for Chordin to co-distribute with the placement of HS in these tissues. In addition, heparin lyase treatment destroyed the Chordin signal in ribs and lung, demonstrating binding of Chordin to be dependent on interactions with endogenous HS in these tissues. The signal detected in skeletal muscles adjacent to the ribs, even after heparin lyase treatment (Fig. 3B), was not due to Chordin binding, but was instead due to nonspecific binding of the primary antibody. A salient feature of Chordin binding in lung was the absence of the Chordin signal in the roughly circular areas of high HS content that correspond to basement membranes surrounding the bronchioles (Fig. 4A). This is illustrated more clearly in Fig. 4B, in which the Chordin signal was seen to be absent from basement membranes surrounding bronchioles and blood vessels, but present in bronchiolar epithelial cells (Fig. 4B, asterisks) and in smooth muscle cells surrounding both bronchioles and blood vessels. The HS-rich circular areas that showed no signal for Chordin were confirmed as basement membranes by detection with antibodies to the basement membrane-specific HSPG perlecan. Additionally, at a higher magnification (Fig. 4C), intense and punctate patterns of the Chordin signal were seen in cells enrolling airway passages, whereas the signal was absent from basement membranes adjacent to those cells.

In a similar fashion, the signal for Chordin was clearly detectable in cells, but absent from basement membranes in two additional tissues in which relatively high levels of Chordin signal were detected: gut (Fig. 5A) and brain (Fig. 5B). Table I summarizes the distribution of Chordin binding and background signals in the tissues that were evaluated.

Chordin Binds Cell-surface HSPGs—Since the signal for bound Chordin in tissues was associated with cellular rather than basement membrane HSPGs (see above), we more closely examined the ability of Chordin to bind cell-surface HSPGs. Toward this end, Chordin was incubated with cultured 101/2 mouse embryo cells, which have previously been employed in culture-based assays of Chordin function (16). As shown in Fig. 6A, Chordin bound 101/2 cell surfaces in an HS-dependent fashion. Such binding also demonstrated the same selectivity for HSPGs as that observed in vivo since Chordin failed to co-localize with perlecan in any of the 101/2 cultures examined.
Such selectivity contrasted with the binding in similar cultures of FGF1, a well-characterized HS-binding growth factor, which not only bound 10t1/2 cell-surface HS, but which co-localized with perlecan as well (Fig. 6A).

To determine the profile of HSPG core proteins associated with 10t1/2 cells, Western blotting of heparin lyase-treated cell layers was performed using mAb 3G10. This approach showed 10t1/2 cells to contain syndecan-1, -2, and -4 and glypicans (Fig. 6B). To begin characterizing which cell-surface HSPGs might be involved in binding, Chordin was incubated with cultured Raji cells, a lymphoblastoid cell line with little or no expression of endogenous cell-surface proteoglycans, or with Raji-S1 cells, a stable line of Raji cells transfected with an expression vector for syndecan-1 (43). As shown in Fig. 6C, although Chordin did not bind to parental Raji cells, it avidly bound to Raji-S1 cells. Similar results were obtained with Raji-S4 cells. Thus, Chordin binds cell-surface HSPGs, but this binding does not appear to be specific to HS chains attached to a single type of HSPG core protein.

Cell-surface HSPGs Are Powerful Regulators of Chordin Antagonism of BMP Signaling and of Cellular Uptake of Chordin—To determine whether interactions with HSPGs might affect the ability of Chordin to antagonize BMP signaling, we transfected M2-10B4 murine marrow stromal cells with a Chordin expression vector and compared the ability of exogenously added BMP-4 to induce osteoblastic differentiation of these precursor cells in standard culture medium (DMEM) or under conditions in which sulfation of HS is blocked (low-sulfate DMEM containing chlorate) (52). Interestingly, Chordin was detected only in the conditioned medium of transfected M2-10B4 cells cultured in low-sulfate DMEM and chlorate (Fig. 7A), consistent with the possibility that secreted Chordin is
trapped at the cell surface by HSPGs under standard growth conditions. In fact, immunofluorescent staining showed transfected M2-10B4 cells grown under normal culture conditions to be associated with high levels of signal for Chordin, including high levels of intracellular Chordin, whereas transfected M2-10B4 cells cultured in low sulfate medium and chlorate were essentially devoid of signal for associated Chordin.

The levels of BMP signaling, as reflected by the levels of osteoblastic differentiation induced in M2-10B4 cells by addition of exogenous BMP-4, were determined by measuring the induced levels of osteoblast-specific AP activity. Importantly, whereas the ability of BMP-4 per se to induce osteoblastic differentiation was unaffected by low sulfate/chlorate treatment, the ability of Chordin to inhibit BMP signaling was totally abrogated under the same conditions (Fig. 7B). Thus, HSPGs appear to play an essential role in the ability of Chordin to modulate BMP signaling. It should be noted that, in control experiments in which 10 mM sulfate was added to chlorate-treated cultures at the same time as chlorate, chlorate treatment had no effect on either the association of Chordin with cells, as detected by immunofluorescence, or the ability of Chordin to inhibit BMP signaling in assays such as that used for Fig. 7B (data not shown). Since addition of 10 mM sulfate allows sulfation of HSPGs to occur in the presence of the concentrations of chlorate used here (52), such controls show the effects of low sulfation/chlorate to occur via sulfation alone and not via some unanticipated effect of chlorate.

The above experiments show HSPGs to be involved in retaining Chordin at the cell surface and in the cellular uptake of Chordin. Such processes should profoundly affect the diffusion of Chordin. In fact, the release of Chordin into the medium of cultures in which sulfation was blocked (Fig. 7A) shows the degree to which HSPGs can affect release of Chordin beyond the cell surface. However, since it is possible that diffusion of Chordin may occur directly from the surface of a Chordin-producing cell to the surfaces of adjacent cells, we fluorescently tagged transfected Chordin-producing M2-10B4 cells, co-cultured them with non-transfected M2-10B4 cells, and then used

| HS site                  | Primary + secondary Abs alone | +60 nM Chordin |
|-------------------------|-------------------------------|---------------|
| Skin                    | –                             | –             |
| Skeletal muscle         | +                             | +             |
| Mast cells              | –                             | –             |
| Gut                     | –                             | +             |
| Rib                     | –                             | +             |
| Lung                    | +                             | +             |
| Liver                   | –                             | –             |
| Submandibular gland     | –                             | –             |
| Atrium                  | –                             | –             |
| Ventricle               | –                             | –             |
| Artery                  | –                             | –             |
| Vein                    | +                             | –             |
| Brain capillary         | +                             | +             |
| Brain                   | –                             | +             |

**Table I**

Summary of exogenous Chordin-HS interactions in various mouse embryonic tissues.

Sectioned embryos were treated either with anti-Chordin primary and secondary antibodies (Abs) alone as a control or with 60 nM exogenous Chordin followed by primary and secondary antibodies. Chordin-HS interactions are denoted by + or +, reflecting the relative binding intensities. Background signal could be detected on Western blots of homogenized muscle samples using these antibodies (W. N. Pappano and D. S. Greenspan, unpublished data).

![Fig. 6. Chordin interacts with cell-surface HSPGs on 10t1/2 fibroblasts.](image)

A, fixed monolayers of 10t1/2 fibroblasts were incubated with 60 nM Chordin (upper left panel) or pretreated with heparinasises I and III, followed by incubation with 60 nM Chordin (upper middle panel). Cells were also incubated with anti-Chordin antibody in the absence of Chordin addition (upper right panel). 10t1/2 cells were also incubated with FGF1 (30 nM) and a fusion protein (100 nM) consisting of the extracellular portion of FGF receptor-1 fused to AP (FR1cAP) following no treatment (lower left panel) or treatment with heparinasises I and III (lower middle panel). The arrow in the lower right panel indicates extracellular matrix HSPG distribution following staining with anti-perlecan antibody. The arrow in the lower left panel indicates FGF1/FR1cAP binding to HS associated with perlecan. B, the 10t1/2 HSPG core protein expression profile was determined following no treatment (–) or treatment with heparinasises I and III (+). C, the binding of Chordin to parental Raji cells (upper left panel), Raji-S1 cells (upper middle panel), or Raji-S4 cells (upper right panel) was investigated following incubation with 60 nM Chordin. Cells were also incubated with anti-Chordin antibody alone (center panels), with anti-syndecan-1 antibody (lower left and middle panels), or with anti-syndecan-4 antibody (lower right panel). Although not shown, parental Raji cells showed no signal when incubated with anti-syndecan-4 antibody.
immunofluorescent staining to determine whether Chordin was associated only with Chordin-producing cells or with adjacent non-producers as well. As shown in Fig. 8, detectable Chordin was associated only with Chordin-producing cells. The results from Figs. 7 and 8 thus indicate that HSPGs profoundly limit the diffusion of Chordin from the cell surface to the extracellular space and from one cell surface to another.

DISCUSSION

Here, we have demonstrated that mammalian Chordin binds heparin in vitro with a high affinity matching those of factors, such as FGFs, that have previously been shown to bind and functionally interact with HSPGs in tissues. Moreover, we have shown that Chordin binds tissues in a manner dependent upon its ability to bind HSPGs. This binding seems limited to cell-surface HSPGs, as basement membranes rich in the HSPG perlecan were not detectably bound. The binding to cell-surface (but not basement membrane) HSPGs denotes specificity in the interactions of Chordin with HSPGs, thus indicating that charge alone is not the sole determinant of Chordin-HSPG binding. We have also shown that Chordin is capable of binding both syndecan-1 and syndecan-4. The latter result indicates that Chordin-HS binding is not limited to a single type of HSPG core protein. Rather, Chordin binding may be specific to a certain type of HS chain, which, in turn, may attach to a variety of core proteins. In this regard, it is of interest to note that binding of FGFs to tissue HSPGs has been shown to vary in response to cell type/tissue-specific differences in HS sulfation patterns (44). Thus, similar differences in HS chains may determine the degree to which Chordin binds different cells and tissues. The issues of which spectrum of cell-surface core proteins and what types of HS side chains may be involved in determining Chordin binding remain to be resolved.

Interestingly, the tissues to which exogenously added Chordin binds most strongly in an HSPG-dependent way (e.g., cells lining the air passageways of the lungs, developing bone, gut, and brain) are those in which we have previously shown Chordin RNA to be expressed at the highest levels during development and in the adult (19, 25, 51). Thus, higher Chordin levels may normally be found localized to the surfaces of cells that secrete Chordin.

Cell-surface HSPGs are integrally involved in modulating the signaling of various growth factors, including FGFs (52, 53), hepatocyte growth factor (54, 55), heparan sulfate-binding epidermal growth factor (56, 57), and vascular endothelial growth factor (58, 59). In addition, the Drosophila glypican dally is required for normal DPP signaling (36), whereas heparin appears to be able to modulate signaling by BMP-2 in embryonic chick limb bud assays (35). Here, we have demonstrated that cell-surface HSPGs bind Chordin and that such interactions serve to powerfully enhance the ability of Chordin to antagonize BMP signaling. Specifically, the ability of Chordin to antagonize BMP signaling was essentially abrogated in M2-10B4 cells via blocking of HSPG sulfation. Although a postulated role for HS binding is the protection of proteins from digestion by endogenous proteinases (60), we have found that heparin did not appreciably protect Chordin from in vitro cleavage by BMP-1, a proteinase responsible for the in vivo proteolysis of Chordin in mammals (20). Thus, a likely interpretation of the various data is that cell-surface HSPGs act to potentiate Chordin antagonism of BMP signaling by concentrating Chordin at the cell surface, where it is optimally placed to limit the binding of BMP-2/4 to cognate cell-surface receptors. Interest-

\[\text{FIG. 7. HSPGs participate in cellular binding and uptake of Chordin and potentiate Chordin antagonism of BMP signaling.} \]

A, M2-10B4 cells transfected either with an empty vector or with a Chordin expression vector were grown in DMEM or in low sulfate (LS) DMEM containing chlorate. Conditioned media were then subjected to Western blot analysis for the presence of Chordin (left panel). Cell layers were subjected to immunofluorescent staining with anti-Chordin antibody (right panels). The insets show fields of cells at lower magnifications, and the higher magnifications show details of cellular staining. B, M2-10B4 cells transfected either with an empty vector or with a Chordin expression construct were incubated in DMEM or in low sulfate DMEM containing 50 mM chlorate in the presence (+) or absence (−) of BMP-4. After 48 h, AP activity, as a measure of BMP signaling, was quantified by reading the absorbance at 405 nm and is given in arbitrary units.

\[\text{W. N. Pappano and D. S. Greenspan, unpublished data.}\]
ingly, chlorate did not affect the levels of BMP signaling in M2-10B4 cells in the absence of Chordin (Fig. 7B). Thus, despite the ability of BMP-4 to bind heparin, sulfated HSPGs may not regulate BMP signaling, in at least some cell types, in the absence of exogenous inhibitors such as Chordin.

Blockage of HSPG sulfation not only abrogated Chordin inhibition of BMP-2/4 signaling, but also decreased the high levels of intracellular Chordin in transfected M2-10B4 cells down to basal levels. The most straightforward interpretation of this effect is that intracellular Chordin in transfected M2-10B4 cells is predominantly Chordin that has been previously secreted and subsequently endocytosed via a mechanism dependent upon cell-surface HSPGs. Previously, Srinivasan et al. (15) demonstrated that uptake of SOG by cells in vivo is a dynamin-dependent mechanism involved in limiting extracellular accumulation of SOG dorsally, thus acting to maintain a proper SOG gradient in *Drosophila* embryos. Our results with the SOG ortholog Chordin are consistent with the roles that HSPGs have been reported to play in this type of uptake and degradation of various extracellular proteins (34), including the BMP antagonist Follistatin (61). It will be of interest to determine whether HSPG facilitates the uptake and degradation of not only Chordin but of Chordin-BMP-2/4 complexes as well or perhaps complexes of Chordin bound to BMP-2 and BMP-4 and their associated type I and II receptors. HSPG potentiation of BMP-2/4 and receptor uptake and degradation would constitute another mechanism whereby HSPG might serve to potentiate the inhibition of BMP signaling, although the data of Fig. 7 show that such a mechanism would be dependent on the presence of Chordin. Chordin bound to cell-surface HSPGs might well have relatively frequent interactions with BMP-2/4-receptor complexes, as both would be limited to two-dimensional diffusion within the same plane.

Larrain et al. (62) have reported that Chordin binds to cell surfaces via integrin α. It seems quite possible that Chordin may bind both cell-surface HSPGs and other cell-surface proteins such as integrins, and no data in this study are contrary to that possibility. However, Larrain et al. also reported that bound Chordin appears to co-localize with structures reminiscent of focal adhesion plaques and with actin filaments underlying the cell surface. Although we did find that Chordin bound nonspecifically to intracellular filaments in permeabilized 10t1/2 cells or to patches of filaments in partially permeabilized 10t1/2 cells, we did not see Chordin localization to adhesion plaques when permeabilization was avoided (data not shown). Thus, perhaps due to differences in technique, we cannot confirm the distribution of cell-surface Chordin binding reported in the previous study.

In addition to the roles of Chordin-HSPG interactions in potentiating the antagonism of BMP signaling by Chordin and cellular uptake of Chordin, another outcome of the strong interactions between Chordin and cell-surface HSPGs appears to be limiting the free diffusion of Chordin. Thus, the combined effects of cell-surface HSPGs on Chordin would be expected to powerfully contribute to shaping and stabilizing fields of BMP inhibition in vivo, with concomitant shaping and stabilizing of
reciprocal fields of BMP signaling. Such interactions may op-
erate both in formation of the embryonic dorsalventral axis and in organogenesis. With regard to the latter, our finding of a
relation between these tissues that produce and those tissues
that bind the highest levels of Chordin suggests that such
binding may help localize peak levels of Chordin to those areas
that synthesize the protein and in this way sharpen the fields
and structures of presumptive organs. Our finding of Chordin
that synthesize the protein and in this way sharpen the fields
secondary dorsal axes in
in antagonizing BMP signaling, TSG is incapable of inducing
completely bound by the surface HSPGs of Chordin-producing
to heparin-Sepharose despite previous work showing
Chordin and
tern whereby fields of BMP signaling are shaped by SOG/
combinations of mammalian TSG
findings that vertebrate TSG, Chordin, and BMP-2/4 form a
recombinant transmembrane domain. This suggests that teth-
ing to cell-surface HSPGs via Chordin. The sys-
ternary complex (24–26) and on the present finding that mouse
findings that indicate that Chordin and BMPs are involved in the point of divergence of the system in widely diverged species.

REFERENCES
1. Wonney, J. M., Rosen, V., Celeste, A. J., Mitsock, L. M., Whitters, M. J., Kriz, R. W., Hewick, R. M., and Wang, E. A. (1988) Science 242, 1528–1534
2. Celeste, A. J., Iannazzi, J. A., Taylor, R. C., Hewick, R. M., Rosen, V., Wang, E. A., and Wonney, J. M. (1990) Proc. Natl. Acad. Sci. U. S. A. 87, 9843–9847
3. Wang, E. A., Rosen, V., D’Alessandro, J. S., Bauduy, M., Cordes, P., Harada, T., Israel, D. I., Hewick, B. M., Kerka, K. M., LaPan, J., Luxenberg, D. P., McQuaid, D., Moutsatsos, I. K., Nove, J., and Wonney, J. M. (1990) Proc. Natl. Acad. Sci. U. S. A. 87, 2220–2224
4. Hogan, B. L. (1996) Genes Dev. 10, 1580–1594
5. Graff, J. M., Thies, R. S., Song, j. j., Celeste, A. J., and Melton, D. A. (1994) Cell 79, 169–179
6. Suzuki, A., Thies, R. S., Yamagi, N., Song, J. J., Woywodt, J. M., Murakami, K., and Ueno, N. (1994) Proc. Natl. Acad. Sci. U. S. A. 91, 10255–10260
7. Hawley, S. H., Woywodt, K., Hashimoto, C., Laurent, M. N., Watabe, T., Blumberg, B. W., and Cho, K. W. Y. (1995) Genes Dev. 9, 2602–2616
8. Cho, K. W. Y., and O’Connor, M. B. (1997) Cell 91, 417–426