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Heparan Sulfate Acts as a Bone Morphogenetic Protein Coreceptor by Facilitating Ligand-induced Receptor Hetero-oligomerization

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Cell surface heparan sulfate (HS) not only binds several major classes of growth factors but also sometimes potentiates their activities—an effect usually termed “coreception.” A view that coreception is due to the stabilization of growth factor–receptor interactions has emerged primarily from studies of the fibroblast growth factors (FGFs). Recent in vivo studies have strongly suggested that HS also plays an important role in regulating signaling by the bone morphogenetic proteins (BMPs). Here, we provide evidence that the mechanism of coreception for BMPs is markedly different from that established for FGFs. First, we demonstrate a direct, stimulatory role for cell surface HS in the immediate signaling activities of BMP2 and BMP4, and we provide evidence that HS–BMP interactions are required for this effect. Next, using several independent assays of ligand binding and receptor assembly, including coimmunoprecipitation, cross-linking, and fluorescence fluctuation microscopy, we show that HS does not affect BMP binding to type I receptor subunits but instead enhances the subsequent recruitment of type II receptor subunits to BMP-type I receptor complexes. This suggests a view of HS as a catalyst of the formation of signaling complexes, rather than as a stabilizer of growth factor binding.

INTRODUCTION

The glycosaminoglycan heparan sulfate (HS) binds many polypeptide growth factors, including fibroblast growth factors (FGFs), Wnts, hedgehogs, transforming growth factor (TGF)-β1 and -β2, heparin-binding members of the epidermal growth factor (EGF) family (e.g., heparin-binding EGF-like growth factor, neuregulins), and hepatocyte growth factor, and it is thought to act as a coreceptor for many of them (Rapraeger et al., 1991; Aviezer and Yayon, 1994; Zioncheck et al., 1995; Lyon et al., 1997; Bellaiche et al., 1998; Kleeff et al., 1998; Tsuda et al., 1999). Most of what is known about mechanisms of HS coreception comes from studies of FGFs. HS binds directly to FGFs and FGF receptors, allowing the formation of stable ternary complexes (Spivak-Kroizman et al., 1994; Plotnikov et al., 1999; Schlessinger et al., 2000) that display increased ligand–receptor affinity and stability (Nugent and Edelman, 1992; Pantoliano et al., 1994; Ibrahimi et al., 2004).

In vivo, most HS is found as a component of heparan sulfate proteoglycans (HSPGs), which include abundant cell surface molecules such as glypicans and syndecans (Lander and Selleck, 2000; Perrimon and Bernfield, 2000). Interestingly, in in vitro assays of FGF signaling, free HS, HS fragments, heparin (a fragmented, highly sulfated variant of HS), or even small-molecule heparin mimetics can all substitute for endogenous HS in promoting receptor binding and signaling. Indeed, the ability to reconstitute coreception with exogenous soluble molecules, at least for FGF signaling, has been crucial in the investigation of coreception (Kruft et al., 1996; Goedger et al., 2008).

Bone morphogenetic proteins (BMPs) belong to the TGF-β superfamily of growth factors and are involved in numerous developmental and physiological processes (e.g., Hogan, 1996; Yoon and Lyons, 2004; Blitz and Cho, 2009). The closely related paralogues BMP2 and BMP4 are among the most widely studied BMPs, and, like most BMPs, they bind strongly to HS (Sampath et al., 1998; Paralkar et al., 1991). Studies in Drosophila indicate that the responses of cells to Decapentaplegic (Dpp), the fly orthologue of BMP2/4, are dependent on HS (Lin and Perrimon, 2002; Bornemann et al., 2004). For example, overexpressing the cell surface HSPG Dally in the larval wing disc causes cell-autonomous increases in Dpp signaling (Fujise et al., 2003). Conversely, groups of cells rendered unable to synthesize HS show marked, cell-autonomous reduction in Dpp signaling (Bornemann et al., 2004). More recently, studies in Xenopus embryos have shown that depletion of the cell surface HSPG syndecan-1 leads to a reduction in BMP-dependent gene expression and patterning (Olivares et al., 2009). BMP signaling also can be disrupted in Xenopus embryos by forced
expression of an endosulfatase that selectively removes 6-O sulfate groups from HS chains (Freeman et al., 2008).

Despite strong in vitro evidence for a stimulatory role for endogenous HS in BMP signaling, the results of in vitro studies have been inconsistent. One study argued that a variant of BMP2 engineered so as not to bind HS is more potent than wild-type BMP2 and also argued that exogenous heparin inhibits BMP activity (Ruppert et al., 1996). In a later study, heparin had the opposite effect on BMP signaling (Takada et al., 2003). Even within a single cell type (mouse C2C12 cells), there are reports showing that heparin can enhance (Zhao et al., 2006) or inhibit (Jiao et al., 2007) BMP2 signaling.

The interpretation of in vitro studies is complicated by several factors, some of which have to do with reliance on long-term assays (lasting hours or days). First, there is considerable evidence that, in addition to acting as a coreceptor, HS affects the bioavailability of growth factors, playing a role in their uptake and destruction, as well as their effective diffusion (Hashimoto et al., 1997; Sperinde and Nugent, 2000; Ohkawara et al., 2002; Belenkaya et al., 2004; Han et al., 2005). In long-term-cultures, such processes may have large effects on the amount and duration of BMP availability. Second, exogenous glycosaminoglycans probably influence processes other than BMP signaling, including signaling by other growth factors, cell adhesion, or even the activities of endogenous BMP antagonists. Third, basing expectations about the effects that reagents such as heparin should have on studies conducted with other classes of growth factors (such as FGFs) presumes an equivalent mechanism of coreception for FGFs and BMPs and may not be appropriate.

For these reasons, we sought to investigate HS coreception under conditions in which BMP signaling is assessed as rapidly and directly as possible, and in which the primary manipulation is the removal or alteration of endogenous HS. The cell lines C2C12 and PC12 were chosen for this work because both have been extensively used not only in the study of BMP signaling but also because considerable information is available about their HSPGs and how to manipulate them. Indeed, both cell lines have been used previously in working out the role of HS in FGF coreception (Damon et al., 1992; Larrain et al., 1998).

MATERIALS AND METHODS

Materials

Materials were purchased from the following companies: DMEM (Mediatech, Herndon, VA), Opti-MEM (Invitrogen, Carlsbad, CA), Hanks’ buffered salt solution (HBSS) (Mediatech), fetal bovine serum (FBS) and heat-inactivated horse serum (Hy-Clone Laboratories, Logan, UT), penicillin-streptomycin solution and L-glutamine (Invitrogen), Na2HPO4 (PerkinElmer Life and Analytical Sciences, Boston, MA); horseradish peroxidase (HRP)-conjugated anti-mouse antibodies (Bio-Rad Laboratories, Hercules, CA), HRP-conjugated anti-rabbit antibodies (GE Healthcare); rat anti-active p38 (anti-phospho-p38 mitogen-activated protein [MAP] kinase [Thr180/Tyr182]), rabbit anti-phospho-Smad1/5/8 (anti-P-Smad), or anti-active p38 antibodies. All films were analyzed by using ImageJ (National Institutes of Health, Bethesda, MD). In cases in which statistical tests were applied to the data, P-Smad and active p38 band intensities were always first.
normalized to total Smad, p38, or β-tubulin band intensities from the same samples.

**BMP Radiiodination, Cell Surface Binding, and Cross-Linking**

Recombinant human BMP4 was radiiodinated using the Chloramine T method (Frollik et al., 1984). BMP4, rather than BMP2, was used because of greater preservation of bioactivity after radiiodination. In brief, 1 μg of BMP4 was diluted in 10 μl of 0.8 M NaONO3, pH 7.5, supplemented with 1 mM of NaCl, and incubated with 0.5 μg of chloramine T for 1 min. To terminate the reaction, 10 μl of 0.62 M acetyl tyrosine, 100 μl of 60 mM KI, and 100 μl of 1 M acetic acid were added. This mixture was desalted on a NAP 5 column (Pharmacia, Peapack, NJ) preequilibrated with 0.1% BSA, 4 mM HCl, and 75 mM NaCl. 125I-labeled BMP4 collected in the void volume was stored at 4°C for up to 4 wk. Recovery was calculated by measuring biological activity of labeled material using a BMP reporter cell line, and calibrated using a standard curve obtained at the same time with unlabeled BMP2.

Binding of radiolabeled BMP4 to untreated or heparitinase-treated C2C12 cells was carried out as described previously (Iwasaki et al., 1995). In brief, cells were seeded in normal growth medium at 5 × 10⁴ cells/dish in 10-cm culture dishes 12–16 h before assay. Cultures were washed twice with 10 ml of serum-free medium and incubated in 5 ml of serum-free medium, containing binding buffer with bivalent BS 3 (6.5 g/mM), 10 ng/ml125I-BMP4 (4.5 × 10⁵ cpm), and 10 ng/ml125I-BMP2 (either room temperature or 4°C for 2 h) added directly and incubated with cells for another 2 h at room temperature on a rocker platform, after which cells were washed twice with HBSS. Binding was performed at room temperature because of the known cold sensitivity of BMP binding to cells (Parlakar et al., 1991; Iwasaki et al., 1995), which we verified in PC12 cells (specific binding of 125I-BMP4 was threefold lower at 4°C than at room temperature). To verify that significant internalization of BMP does not occur during room temperature incubation, 6 × 10⁴ PC12 cells were incubated in serum-free medium at 37°C for 1 h and then at 4°C for 10 min. Samples were then subjected to 10% SDS-PAGE for analysis.

For cross-linking, freshly prepared BS 3 (in 25 mM HEPES-buffered saline, pH 7.5) was used and incubated with 100 μM NaF on ice for 5 min. Lysates were collected by centrifugation for 10 min at 14,000 × g. Supernatants were subjected to immunoprecipitation with anti-HA (1:150) or anti-myc (1:150) antibodies at 4°C overnight. After washing three times with ice-cold PBS, the cells were lysed (in 10 mM Tris-HCl, pH 7.5, with 100 mM NaCl, 1 mM EDTA, 0.5% Triton X-100, 1 mM PMSF, 5 μg/ml aprotinin, 1 μg/ml pepstatin A, 1 μg/ml leupeptin, 1 mM NaVO₄, and 50 mM NaF) on ice for 5 min. Lysates were collected by centrifugation for 10 min at 14,000 × g. Supernatants were subjected to immunoprecipitation with anti-HA (1:150) or anti-myc (1:150) antibodies at 4°C overnight. After washing three times with ice-cold PBS, the cells were lysed (in 10 mM Tris-HCl, pH 7.5, with 100 mM NaCl, 1 mM EDTA, 0.5% Triton X-100, 1 mM PMSF, 5 μg/ml aprotinin, 1 μg/ml pepstatin A, 1 μg/ml leupeptin, 1% Triton X-100, 1% sodium deoxycholate, and 0.1% SDS, and boiling in 2× SDS-PAGE sample buffer for 10 min. Samples were then subjected to 10% SDS-PAGE for analysis. The gels were fixed, dried, analyzed by a Phosphorimager (GE Healthcare) for phosphorylation and p38 activation was reduced at every time point (Iwasaki et al., 1999; Gallea et al., 2001). To test for a role for HS in these events, we incubated C2C12 and PC12 cells for 1 h with heparitinase (heparin lyase III, 25 U/ml) and incubated with 10 ng/ml125I-BMP4 (4.5 × 10⁵ cpm). These were incubated in serum-free medium at 37°C for 1 h and then at 4°C for 10 min. Samples were then subjected to 10% SDS-PAGE for analysis. The gels were fixed, dried, analyzed by a Phosphorimager (GE Healthcare) using the ImageQuant program.

**Laser Scanning Confocal Microscopy and Number-and-Brightness Analysis**

An Olympus FV1000 laser scanning confocal microscope with a 60×, 1.2 numerical aperture water objective (Olympus, Tokyo, Japan) was used, with data collected in photon counting mode. For each cell imaged, 200 frames (256 × 256 pixels) were collected (scan speed, 10 μs/pixel). The corresponding line scan data were collected as the frames were acquired. For EYFP expression, the 515 nm line of the argon ion laser was used, at a laser power of 0.5%. Analysis was carried out using Global S for Images (SimcFS; Iwasaki et al., 1999; Vinals et al., 2002). Three-dimensional images were obtained by confocal microscopy and 3D visualization. The software used for 3D visualization was MetaMorph (Universal Imaging Corp.). In brief, cells were incubated with heparitinase, the kinetic profiles were very similar, but the amount of Smad phosphorylation and p38 activation was reduced at every time point (Figure 1B and C, and Supplemental Figure S2).

**RESULTS**

**Heparitinase Diminishes Responses of Cultured Cells to BMP2**

The initial, and most direct, signal transduction event in BMP signaling is the phosphorylation of cytoplasmic Smads 1/5/8 by the activated BMP receptor complex. On a similar time scale, receptor activation also leads to activation of the p38MAPK pathway (Iwasaki et al., 1999; Gallea et al., 2001). When cells were pretreated with heparitinase, the kinetics of BMP-induced Smad phosphorylation and p38 activation almost completely abolished, as in the untreated samples (Figure 1A). A marked decrease of BMP4-induced Smad phosphorylation was also observed in heparitinase-treated cells (Supplemental Figure 1A). In the absence of heparitinase, the kinetics of BMP-induced Smad phosphorylation and p38 activation in both PC12 and C2C12 cells were similar to what has been reported by other groups, peaking at ~60 min then gradually declining (Iwasaki et al., 1999; Vinals et al., 2002). In the absence of heparitinase, the kinetics of BMP-induced Smad phosphorylation and p38 activation in both PC12 and C2C12 cells were similar to what has been reported by other groups, peaking at ~60 min then gradually declining (Iwasaki et al., 1999; Vinals et al., 2002).
removal of HS does not merely delay BMP signaling, it leads to a persistent reduction in overall signaling. As shown in Figure 1, D and E, with heparitinase-treated cells, the addition of heparin—up to 100 μg/ml—neither rescued BMP-induced initial signaling nor reduced it any further. Interestingly, addition of supramaximal amounts of BMP2 also did not restore either p38 activation or Smad phosphorylation in heparitinase-treated cells; indeed, the BMP2 concentration that produced half-maximal signaling (BMP2 concentration that produced half-maximal signaling) was affected by heparitinase treatment (*p < 0.01; t test). Data are from triplicate cultures for each condition; and band intensities were quantified. Data are from triplicate cultures for each condition; and band intensities were quantified.
Thus, heparitinase treatment affects BMP signaling, not p38 activation per se.

**Blockade of Sulfation Diminishes Responses of Cultured Cells to BMP2**

Chlorate, a competitive inhibitor of ATP-sulfurylase, blocks glycosaminoglycan sulfation (Baeuerle and Huttner, 1986) and can thus be used to inhibit the functions of HS, which generally depend upon the presence of sulfated structures (Turnbull et al., 2001; Esko and Selleck, 2002; Powell et al., 2004). Conditions for the use of chlorate to block HS sulfation in C2C12 and PC12 cells have been reported previously (Baeuerle and Huttner, 1986; Melo et al., 1996; Jiao et al., 2007). As shown in Supplemental Figure S3, culture of both cell types under these conditions did not affect overall levels of p38 or Smads 1/5/8 nor did it cause p38 or Smad phosphorylation. However, chlorate-treated cells exhibited significantly reduced activation of p38 and Smad by BMP2 (5 ng/ml for 1 h; Figure 1, I and J). Together, the results in Figure 1 indicate that endogenous HS plays a significant role in potentiating BMP signaling.

**A Non-heparin Binding BMP2 Variant Is Less Sensitive to Removal of HS**

The HS-binding domain of BMP2 has been mapped to its N-terminal 17 amino acids, seven of which are positively charged (Ruppert et al., 1996). Ruppert et al. (1996) engineered a variant form that they named EHBMP2 and that replaces the first 12 residues of mature BMP2 with a homologous sequence from the N terminus of human interleukin-2 (Figure 2A). EHBMP2 is active in BMP signaling but fails to bind heparin with detectable affinity. To investigate whether the heparin-binding domain of BMP2 participates in signaling, we tested whether signaling by EHBMP2 is sensitive to removal of HS chains.

As shown in Figure 2B, we found EHBMP2 to be equivalent in potency to BMP2 on untreated cells. Interestingly, the p38 and P-Smad responses elicited by EHBMP2 in C2C12 cells were unaffected by heparitinase (Figure 2, C–E). Similar experiments were carried out with chlorate-treated C2C12 and PC12 cells, in which Smad phosphorylation and p38 activation were both monitored. As shown in Figure 3, signaling induced by EHBMP2 was highly resistant to chlorate, although with sufficiently high chlorate concentrations it did eventually decrease. Because prolonged chlorate exposure can be toxic at high doses, we cannot be confident that the loss of measured signaling at high chlorate levels is a specific effect of the loss of HS.

Overall, the data in Figures 2 and 3 imply that the sensitivity of BMP2 signaling to the loss of cellular HS is critically dependent on BMP2’s ability to bind HS. Curiously, the data also tell us that HS–BMP interaction is not, in itself, necessary for signaling, because EHBMP2 signals with normal potency on untreated cells (Figure 2B). The implications of this result will be discussed later (see Discussion).

**HS Affects the Binding of BMP to Type II, but Not Type I, Receptor Subunits**

BMP signaling complexes are heteromeric, containing type I and type II receptor subunits (ten Dijke et al., 1994; Liu et al., 1995). Typically, BMPs bind type I receptors with high affinity and are thought to associate first with these molecules, subsequently recruiting the lower affinity type II receptors (reviewed by ten Dijke et al., 1996; Shi and Massague, 2003). To test whether HS plays a role in the binding of BMP to any of its receptors, BMP4 was radioiodinated, and allowed to bind for 2 h at room temperature to C2C12 cells that were transiently transfected with epitope-tagged type I receptor (BMPRIA, HA-tagged), stably transfected with epitope-tagged type II receptor (BMPRII, myc-tagged), or both. After washing, the cells were subjected to cross-linking with 1 mM BS3, lysed, immunoprecipitated with antibodies to HA or myc, and analyzed by reducing SDS-PAGE and autoradiography.

In cells transfected only with BMPRIA, and subjected to the above-mentioned procedure, the anti-HA antibody specifically immunoprecipitated several proteins (Figure 4A, arrows). A band with apparent molecular mass of 78 kDa...
(arrow 3) corresponds to the expected size of a cross-linked BMP-BMPRIA complex. Other bands at 18 kDa (arrow 1) and 36 kDa (arrow 2), correspond to the sizes of free BMP monomer and cross-linked BMP dimer, respectively (specific precipitation of free BMP occurs in these experiments because receptor dissociation is sufficiently slow for BMPs that considerable ligand can remain bound to receptors through cell lysis and immunoprecipitation, even without chemical cross-linking). As Figure 4A shows, the intensities of these bands were unaffected by pretreatment of cells with heparitinase, under the same conditions that strongly inhibited BMP signaling.

In contrast, Figure 4B shows the results when radioiodinated BMP4 was bound to cells stably transfected with BMPRIA, or with both BMPRIA and BMPRII, and immunoprecipitation carried out using anti-myc antibodies (which recognize tagged BMPRII). Arrows mark the expected positions of free BMP (18 kDa, arrow 1), as well as BMP cross-linked to type I receptor (arrow 2), type II receptor (arrow 3) or higher order complexes (arrow 4).

As in Figure 4A, immunoprecipitation of type II receptors specifically brought down a significant amount of free BMP (arrow 1). In addition, besides bringing down bands corresponding to complexes containing BMP and type II receptors, immunoprecipitation of type II receptors also appeared to bring down a significant amount of BMP-type I receptor complexes. Presumably both results reflect the fact that many highly stable BMP-type I receptor-type II receptor heteromeric complexes remain associated even after cells are lysed and therefore stay together during immunoprecipitation. Note also that the amounts of all specifically immunoprecipitated bands are higher in cells that were transfected with type I receptors, which suggests that levels of endogenous type I receptors are limiting for formation of BMP-type II receptor complexes.

As quantified in Figure 4, C and D, treatment of cells with heparitinase resulted in significant reductions in all cross-linked bands. Together, the data in Figure 4 imply that the initial association of BMPs with type I receptors is HS independent, but the subsequent assembly of a signaling complex including type II receptors is HS sensitive. To test this idea directly, C2C12 cells expressing both HA-tagged BMPRIA and myc-tagged BMPRII were treated with or without heparitinase for 1 h, exposed to unlabeled BMP2 for 2 h, and then subjected to cross-linking with 1 mM BS3. Lysates were immunoprecipitated with anti-HA antibodies, and precipitates were then subjected to SDS-PAGE and immunoblotting using anti-myc antibody. In principle, the amount of myc-BMPRII visualized should provide an indication of the amount of type II receptor that is associated with type I receptor.

As shown in Figure 5A, precipitation of BMPRIA brought down a small amount of BMPRII (arrow) even in cells not exposed to BMP (lanes 1–3 and 10–12); this agrees with a previous report that small amounts of preformed BMP receptor complexes are present on cells (Nohe et al., 2002). In response to treatment with BMP2, a much larger amount of BMPRII was brought down, demonstrating that BMP induces the association of type I and II receptors. In addition, immunoprecipitated bands of higher molecular weight can be observed in such samples (lanes 4–6 and 7–9), particularly with longer exposures of the same blot (Figure 5B). Bands marked with arrows numbered 1, 2, and 3 are at the appropriate molecular sizes for BMPRII, BMPRII cross-linked to BMP2, and higher molecular weight products (possibly BMPRII cross-linked to BMPRIA and BMP2), respectively.

When cells treated with BMP2 were pretreated with heparitinase, a marked decrease in the intensity of all bands was observed (lanes 7–9; Figure 5, A and B). In contrast, the amount of BMPRII that was coprecipitated in a ligand-independent manner was not affected. These results indicate that BMP-induced formation of heteromeric receptor com-
plexes is markedly reduced by the removal of cell surface HS.

**HS Facilitates BMP2-induced Type II Receptor Dimerization on Intact, Living Cells**

The predominant mechanism by which BMP induces the formation of signaling complexes is thought to be the addition of two type II receptor subunits to a complex involving BMP and two type I receptors (Gilboa et al., 2000; Greenwald et al., 2003; Nickel et al., 2009). This could occur either by the stepwise addition of individual type II receptors, or by the addition of a single preformed type II receptor homodimer (the latter mechanism being suggested by the observation that type II receptors can homodimerize in the absence of ligand; (Gilboa et al., 2000)). Because one of these two assembly pathways is likely to be faster than the other, we realized that anything that affected ligand-independent homodimerization of type II receptors might indirectly affect the overall rate of signaling complex formation. To see whether changes in type II receptor homodimerization play a significant role in the effects of HS on BMP receptor complex assembly, as well as to obtain independent confirmation of the cross-linking and coimmunoprecipitation results (Figures 4 and 5), we used a variation of fluorescence correlation microscopy to visualize type II receptors in live cells.

**PC12 cells stably expressing BMPRII that was cytoplasmically tagged with an EYFP were treated with or without heparitinase, exposed to BMP2 (or no growth factor) for 2 h, and then confocal fluorescence images were acquired.** For each of 30 cells in every treatment condition, a stack of 200 images was collected. Pixel statistics were analyzed by N&B (Digman et al., 2008), wherein an apparent brightness for each pixel is calculated as the ratio of the variance to the mean of the intensities, at that pixel, in the image series (see Materials and Methods). Assuming that intensity fluctuations reflect the random movements of a quantized amount of fluorescence in and out each pixel, N&B theory states that the amount by which excess intensity (the expected apparent brightness for an immobile source) yields the brightness of the quantum, also known as true molecular brightness (in units of photons/s/molecule). The average aggregation state of mobile fluorescent molecules in any pixel can thus be determined by normalizing observed true brightness to the true brightness obtained for a known monomeric form of the same fluorophore.

**Representative data are shown in Figure 6, A to F. Data in A–C were obtained from a BMPRII-EYFP–expressing cell**
not exposed to any factor; data in D–F were from a cell exposed to BMP2 (10 ng/ml) for 2 h. A and D show the average fluorescence intensity maps for the two cells. B and E are scatter plots of apparent $B$ versus mean intensity for each pixel. A calculation of average $B$ was made for all pixels with intensities above an arbitrary threshold (red box, B and E). As shown in C–F, such pixels (shown in red) correspond closely with the location of the plasma membranes of the two cells, as would be expected for a cell surface receptor.

Figure 6G plots true brightness measurements for 30 independent cells in each of four categories (untreated, BMP2 treated, heparitinase treated, and heparitinase and BMP2 treated), as well as for control PC12 cells that expressed either of two monomeric standards: soluble EYFP (which is localized to the cytoplasm), or a fusion of EYFP to the palmitoylation sequence of GAP43 (which localizes it to the plasma membrane; Zuber et al., 1989). As summarized in Figure 6H, the average aggregation state of BMPRII-EYFP in untreated PC12 cells was slightly but not significantly higher than that of either monomeric control. Thus, if there are predimerized BMPRII molecules on the surfaces of these cells, they are likely present in low amounts. In contrast, when such cells were treated with BMP2, the average aggregation state of BMPRII-EYFP molecules significantly increased, by a factor of ~1.5. When such cells were pretreated with heparitinase, however, BMP2 exposure caused only a 1.2-fold increase in aggregation. Heparitinase treatment alone had no significant effect (Figure 6, G and H).

Because these cells contain endogenous, unlabeled BMPRII in addition to EYFP-tagged BMPRII, we cannot infer absolute stoichiometries of receptor assemblies from the data (e.g., a normalized $z$ value of 1.7 could mean that 70% of all receptors are dimers and 30% monomers; or that 100% are dimerized but 30% of the time tagged receptors are paired with unlabeled endogenous receptors). Nevertheless, the data clearly show that BMP2 causes considerable aggregation of type II receptors. Presumably this reflects the recruitment of type II receptors into heterotetrameric complexes, i.e., signaling complexes that contain two type I and two type II receptors. The data clearly show that, much, but not all, of this recruitment is blocked by heparitinase. Furthermore, they also show that to the extent that preformed, ligand-independent dimers of BMPRII do exist on these cells, their levels are not noticeably altered by heparitinase treatment. This strongly suggests that HS acts by facilitating the recruitment of type II receptors to ligand-type I receptor complexes.

The BMP2 HS-Binding Domain May Impede Association with Type II Receptors

Crystal structures suggest that heteromeric BMP-type I receptor-type II receptor complexes are held together through interfaces between BMP ligands and each of the two receptor subunit types; no direct contacts between receptor ectodomains are observed (Allendorph et al., 2006; Supplemental Figure S4). This suggests that recruitment of type II receptors is mediated by the exposed, type II-receptor-binding domains of BMPs. Indeed, free BMPs can bind directly to the extracellular domains of type II receptors, albeit with much lower affinity than to type I receptors (reviewed by Nickel et al., 2009).

When we used surface plasmon resonance to examine such binding (Figure 7), we noticed that EHBMP2—the BMP2 variant that lacks its HS-binding domain (Figures 2 and 3)—associates with BMPRII ectodomains slightly faster than wild-type BMP2 but that it dissociates at about the same rate (Figure 7, A and B, and Table 1). For type I
DISCUSSION

A Novel Mechanism of Coreception

HS has been linked to regulation of signaling by multiple growth factors and morphogens, through effects collectively termed “coreception” (Bernfield et al., 1999; Kirkbride et al., 2005). Here, we identify a mechanism for HS coreception in BMP signaling that differs from what has been proposed for HS in other growth factor pathways. With FGFs in particular, the primary mechanism of HS action is thought to be stabilization (reduced dissociation) of growth factor–receptor complexes (Fantoniano et al., 1994; Ibrahimi et al., 2004). HS also has been proposed to affect the turnover of FGF–receptor complexes (Roghani and Moscatelli, 1992; Sperinde and Nugent, 1998), and the rate at which cells capture FGFs (Lander, 1999).

In contrast, the data here indicate that HS plays no role in the binding and capture of BMPs by their type I receptors, but instead influences the formation of signaling complexes through the recruitment of type II receptors to occupied type I receptors. This effect requires an intact heparin-binding domain in the BMP, and is not rescued by soluble heparin. This model is supported by experiments with two BMP-responsive cell lines (PC12 and C2C12), two methods for depleting cells of HS (heparitinase and chlorate), two methods for measuring BMP binding (Figure 4) and receptor assembly (Figure 5), additional support can be found in the dose–response relationship in Figure 1, F and G, and the effect of receptor ectodomains, the association and dissociation kinetics of EHBMP2 and wild-type BMP2 were indistinguishable (Figure 7, C and D, and Table 1) and also in agreement with published values (Natsume et al., 1997; Kirsch et al., 2000; Heinecke et al., 2009).

These data indicate that the heparin-binding domain of BMP2 does not influence its initial association with type I receptors, which is in good agreement with the result that HS depletion had no effect on cross-linking of BMPs to type I receptors (Figure 4A). In contrast, the slightly faster binding of EHBMP2 to BMPRII suggests that the heparin-binding domain of BMP2 might actually impede binding to type II receptors. This raises the interesting possibility that the BMP heparin-binding domain is an “autoinhibitory” domain and that the function of cell surface HS is to neutralize it. Although such a model is only a speculation at this point, it does provide a simple explanation for why EHBMP2, which lacks this domain, is as potent as wild-type BMP2, and independent of HS for function (Figures 2 and 3).
Table 1. Kinetics of binding of BMP2 and EHBMP2 to type I and type II BMP receptor ectodomains

<table>
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<th>Ectodomain</th>
<th>$k_{on}$ (s$^{-1}$ M$^{-1}$)</th>
<th>$k_{off}$ (s$^{-1}$)</th>
<th>$K_D$ (nM)</th>
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<tr>
<td>BMPRII-Fc</td>
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<td>BMP2</td>
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Means ± SD of association and dissociation rate constants, derived from surface plasmon resonance measurement of binding of various concentrations of BMP2 or EHBMP2 (2–2000 nM for RI; 0.2–100 nM for RII) to immobilized BMPRII-Fc and BMPRIA-Fc. The apparent dissociation constant ($K_D$) was calculated from the ratio $k_{off}/k_{on}$. At least two independent sensorgrams were obtained at every concentration tests. For representative data, see Figure 7.

if the rate of disassembly of heteromeric signaling complexes ($k_d$) is negligible (a reasonable assumption given the extraordinary stability of BMP–receptor signaling complexes; Iwasaki et al., 1995; Liu et al., 1995; Greenwald et al., 2003), this scheme may be viewed as a Michaelis–Menten process,

$$BMP + RI + RII ⇄ BMP-RI + RII \overset{k_1}{\underset{k_2}{\rightleftharpoons}} BMP-RII$$

in which $S$ is BMP, $k_i$ is $k_i[RI]$, $S^*$ is BMP-RI, $k_i$ is $k_i[RII]$, and $P$ is BMP-RII. According to this scheme, the two most readily observed measures of BMP signaling—the maximal rate of signaling complex production and the $EC_{50}$ (the BMP concentration at which signaling is half-maximal)—should depend only on the quantities $k_i$ and $(k_2 + k_3)/k_1$, respectively (analogous to $v$ for enzymatic reactions). Moreover, if $k_3 \gg k_2$, i.e., if BMP binding to type I receptors
equilibrates on a faster time scale than the recruitment of type II receptors, then $E_{C0} = k_r/k'_s$. Evidence that type II receptor recruitment is indeed the rate-limiting step in signaling complex assembly comes from direct measurements of $k_r$ (Table 1; Wrana et al., 1994; Liu et al., 1995; Natsume et al., 1997; Kirsch et al., 2000; Heinecke et al., 2009); the results of mutational studies (Nickel et al., 2009; and the marked cold sensitivity of the assembly of high-affinity BMP–receptor complexes (Paralkar et al., 1991; Iwasaki et al., 1995), the latter observation being consistent with a rate-limiting step that requires diffusion within a lipid membrane.

According to this analysis, a process that accelerated the recruitment of type II receptors (increased $k'_s$) to BMP–RI complexes, but had no effect on the parameters of interaction of BMPs with type I receptors ($k_2$, and $k_3$), would be expected to increase maximal signaling (proportional to $k_2$) but leave the $E_{C0}$ ($k_2/k_3$) unchanged. This is, of course, precisely what Figure 1, F and G, shows to be the case for HS (and precisely what is not observed in studies of coreception for FGFs; Rapraeger et al., 1991; Yayon et al., 1991; Lin et al., 1999). A more detailed analysis can be carried out in which the two binding sites represented by each BMP dimer and the four recruited receptor subunits are represented explicitly, but the overall conclusions are similar (data not shown). Thus, the data in Figure 1, F and G, themselves lend support to a coreceptor mechanism based on accelerated, intramembranous assembly of heteromeric receptor complexes.

Interestingly, because the term $k'_s$ stands for the product of a rate constant multiplied by the concentration of type II receptors, it follows that at sufficiently high type II receptor concentrations (such that $k'_s \gg k_2$), the rate-limiting step in signaling complex assembly might shift to being the step of initial capture of BMPs by type I receptors, which would in turn render HS ineffective as a coreceptor. Although we do not know whether sufficiently high levels of type II receptor expression occur in vivo, this possibility suggests a way in which the magnitude of HS coreceptor effects in BMP signaling might vary considerably from one cell type to another.

**What Is the Structural Basis for Coreception?**

In its role in regulating blood coagulation, HS is thought to act as a binding template, or scaffold, accelerating the interactions of HS-binding proteins (de Agostini et al., 1990; Rosenberg et al., 1997). Studies of FGFs support a similar model for coreception, as both ligand and receptor bind HS, and complexes containing all three can be cocrystallized (Pellegrini et al., 2000; Schlessinger et al., 2000). In BMP coreception, however, although a direct interaction between ligand and HS seems to be required, evidence for direct receptor–HS interaction is slim. In vitro measurements have suggested that heparin may bind BMPRI and BMPRII receptors very weakly (Kanzaki et al., 2008), but in the three-dimensional structures of the extracellular domains of type I and type II receptors (Greenwald et al., 2003; Allendorph et al., 2006; Weber et al., 2007) the substantial clusters of positive charge seen in most proteins with physiologically significant HS–interactions (reviewed by Capila and Linhardt, 2002) are not observed.

Two other observations point away from a template model for HS action in BMP coreception: First, soluble heparin fails to rescue loss of BMP signaling in HS-depleted cells (Figure 1, D and E; these results also argue against a model of HS-induced conformation change in BMPs). Second, we observed that EHBMP2, which lacks a HS-binding domain and is no longer sensitive to HS depletion, is equal in potency and maximal effect to wild-type BMP2 (Figure 2B).

Although we cannot rule out the possibility that the relative behaviors of BMP2 versus EHBMP2 are influenced by differences in the way these ligands were prepared (BMP2 was prepared in mammalian cells, whereas the EHBMP2 used here was produced in bacteria), the in vitro receptor-binding rate constants that we obtained for these molecules (Table 1 and Figure 7) agree well with those obtained when both are prepared from the same source (Knaus and Sebald, 2001). For this reason, we suggest that coreception might result from the blockade of an autoinhibitory activity residing in the BMP2 HS-binding domain. However, as this domain is consistently not resolved in crystallographic studies, we can only speculate regarding structural mechanisms for such autoinhibition (Supplemental Figure S4). Further clarification will no doubt require additional experimental work (e.g., directed mutagenesis studies).

**Is HS a Catalytic Coreceptor?**

In the prevailing model of FGF signaling, HS is seen as a stable, structural component of the signaling complex. This view is supported by the known ability of heparin and HS to form stable, high-affinity complexes with FGFs and FGF receptors (Spivak-Kroizman et al., 1994; Chang et al., 2000; Schlessinger et al., 2000). In BMP signaling, we have so far been unable to obtain any evidence for stable association of HS with BMP–receptor complexes (Kuo and Lander, unpublished observations). Although we cannot draw firm conclusions from negative results, the possibility exists that the role of HS is essentially catalytic, promoting heteromeric receptor assembly but not remaining stably associated with signaling complexes. Indeed, a general view of HS as a “catalyst of molecular encounter” has been proposed frequently in the past, and not just in regard to growth factor signaling (Lander, 1998; Park et al., 2000).

Interestingly, one of the best known examples of coreception based on catalysis of receptor assembly occurs within the same growth factor superfamily as the BMPs. TGF–βs assemble their signaling complexes through initial binding to type II receptors, followed by recruitment of type I receptors. The coreceptor protein betaglycan binds TGF–βs and facilitates their binding to type II receptors but then is subsequently displaced upon the binding of type I receptors (Lopez-Casillas et al., 1993, 1994). In addition, betaglycan often carries HS chains, and although HS is not required for the interaction of TGF–βs or BMP2 with the betaglycan polypeptide (Lopez-Casillas et al., 1994; Kirkbride et al., 2008), HS may well play independent roles in coreception, similar to those described here. Indeed, we have consistently observed inhibitory effects of heparinase on TGF–β1-stimulated p38 activation in C2C12 cells (unpublished data).

**Noncoreceptor Roles of HS**

A variety of findings suggest that HS also regulates BMP signaling through mechanisms other than coreception. For example, HS can facilitate internalization and degradation of BMPs (Jiao et al., 2007), potentially explaining why removal of the HS-binding domain from BMP4 gives it greater potency and a longer spatial range of action in Xenopus embryos (Ohkawara et al., 2002). This also may explain why EHBMP2 shows greater biological potency than BMP2 in long-term in vitro studies (Ruppert et al., 1996) but not in the short-term assays performed here (Figure 2B). Moreover, the ability of exogenous heparin to interfere with BMP uptake by cell surface HS (Zhao et al., 2006) probably explains in vitro results in which heparin has been reported to stimulate BMP activity (Fisher et al., 2006; Zhao et al., 2006).
At the same time, recent results in Drosophila have been interpreted as implying that cell surface HS “stabilizes” Dpp, blocking its degradation (Akiyama et al., 2008). Thus, whether endogenous HS acts primarily to deplete or protect BMPs may be a matter of tissue context.

HS also may influence BMP function through interactions with secreted BMP antagonists. For example, cell surface HS both potentiates the antagonism of BMPs by chordin and mediates chordin uptake (Jasuja et al., 2004). The BMP inhibitor noggin associates with cell surfaces through binding to HS, and is functionally active there (Paine-Saunders et al., 2002; Viviano et al., 2004). In Drosophila, the chordin-related antagonist cross-veinless-2 also associates with cell surfaces via interaction with HS (Serpe et al., 2008).

Given the wide variety of ways in which HS can influence BMP function—as a coreceptor, as a regulator of ligand uptake and degradation, and as a regulator of antagonist localization and function—it is perhaps not surprising that investigations into the role of HS in BMP signaling have sometimes produced contradictory results. In the end, we believe that the multiplicity of potential HS effects reflects a deep and widespread involvement of HSPGs in the regulation of BMP function in vivo.

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