**A concentration-dependent endocytic trap and sink mechanism converts Bmpr from an activator to an inhibitor of Bmp signaling**

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Bmpr, which is orthologous to *Drosophila melanogaster* crossveinless 2, is a secreted factor that regulates Bmp activity in a tissue- and stage-dependent manner. Both pro- and anti-Bmp activities have been postulated for Bmpr, although the molecular mechanisms through which Bmpr affects Bmp signaling are unclear. In this paper, we demonstrate that as molar concentrations of Bmpr exceed Bmp4, Bmpr dynamically switches from an activator to an inhibitor of Bmp4 signaling. Inhibition of Bmp4 through a novel endocytic trap-and-sink mechanism leads to the efficient degradation of Bmpr and Bmp4 by the lysosome. Bmpr-mediated internalization of Bmp4 reduces the duration and magnitude of Bmp4-dependent Smad signaling. We also determined that Noggin and Gremlin, but not Chordin, trigger endocytosis of Bmps. This endocytic transport pathway expands the extracellular roles of selective Bmp modulators to include intracellular regulation. This dosage-dependent molecular switch resolves discordances among studies that examine how Bmpr regulates Bmp activity and has broad implications for Bmp signal regulation by secreted mediators.

**Introduction**

Bmp pathways are tightly regulated at multiple levels of signaling to allow for diverse biological function. The Bmp signals that initiate target cell activation are strongly influenced by extracellular Bmp modulators (for review see Balemans and Van Hul, 2002). The extracellular cues that target these downstream Bmp signaling components to distinct endocytic pathways remain unclear. Extracellular Bmp modulators, including Bmpr (Bmp-binding endothelial cell precursor-derived regulator), a member of the Kieln-Chordin–related protein subfamily, are an important component of Bmp regulation as they help control the boundaries and sensitivity of Bmp signaling during many aspects of development (Michos et al., 2004; Rentzsch et al., 2006; Choi et al., 2007). Until recently, disparate reports of Bmpr serving both as a pro- and anti-Bmp factor failed to support a model that explained the dual nature of Bmpr activity (Conley et al., 2000; Moser et al., 2003; Binnerts et al., 2004; Coles et al., 2004; Kamimura et al., 2004; Ralston and Blair, 2005; Ikeya et al., 2006; Rentzsch et al., 2006; Serpe et al., 2008). Simple models of the biological role of Bmpr do not resolve the dissonance identified in these divergent systems.

Recent data provide support for a model in which the activity of Cv-2 is biphasic with low levels of Cv-2 promoting and high levels inhibiting signaling during the formation of the crossveins in the *Drosophila melanogaster* wing. In this system, Cv-2 was found to selectively interact with Bmp ligands and receptors in a concentration-dependent manner (Serpe et al., 2008). The present study provides further evidence that Bmpr behaves in a concentration-dependent manner. Bmpr enhances Bmp4-mediated Smad activation at molar concentrations below that of Bmp4 in endothelial cells. Alternatively, when Bmpr concentrations exceed those of Bmp4, they internalize interdependently into an endocytic shuttle to the lysosome for their dissolution. Interestingly, we found that Noggin and Gremlin, but not Chordin, can also trigger Bmp endocytosis, suggesting...
active transport? Is this phenomenon selective to certain cell types? Which intracellular pathway does Bmper traffic through? What are the structural requirements of Bmper protein that mediate internalization? How does Bmper internalization affect Bmp signaling?

The mouse embryo-derived endothelial cell line (mouse endothelial cell [MEC]) expresses all of the components required for appropriate Bmp signaling (Valdimarsdottir et al., 2002). To assess whether Bmper internalizes by an active process or through passive flow, we compared MECs treated with 6 nM of recombinant Bmper at temperatures that promote (37 °C) and inhibit (4 °C) active transport (Fig. 1a). Bmper is processed into two fragments that remain associated by disulfide bonds (Binnerts et al., 2004; Rentzsch et al., 2006); both the N- and C-terminal fragments of Bmper were detected by Western analysis.

**Results**

**Endocytic internalization of extracellular Bmper**

While probing for the effects of Bmper on Bmp6-mediated Cox2 induction (Ren et al., 2007), we discovered that recombinant Bmper (6 nM) was internalized to the cytosol when added to the culture media of endothelial cells (Fig. 1a). As a first step to determine whether this observation represented a biologically relevant phenomenon of internalization, we answered the following questions: does Bmper cross the membrane of endothelial cells and, if so, is it through passive absorption or active transport? Is this phenomenon selective to certain cell types? Which intracellular pathway does Bmper traffic through? What are the structural requirements of Bmper protein that mediate internalization? How does Bmper internalization affect Bmp signaling?

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when BMECs treated with Bmp per were chased with recombinant Bmp per (Fig. 1g). When MECs were treated with recombinant Bmp per but with cold media or cold media containing chloroquine for 2 h, and then chased without recombinant Bmp per but with cold media or cold media containing chloroquine for an additional 2 h (Fig. 2a). Both recombinant Bmp per (6 nM) and Bmp per from HEK-293 conditioned media (6 nM) were internalized similarly by MECs during the pulse. To determine if Bmp per internalizes into an endosomal transport pathway, we disrupted acidic endosomal transport during the chase phase of this experiment. Internalized Bmp per was eliminated by MECs during the 2-h cold chase but, in striking contrast, Bmp per levels were sustained in these cells in the presence of chloroquine. A kinetic analysis indicated that the half-life of intracellular Bmp per was ~30 min and that steady-state levels were almost completely stabilized in cells treated with chloroquine (Fig. 2b and c). These data provide further evidence of Bmp per reentry from the extracellular space and indicate that Bmp per is subsequently targeted for lysosomal degradation through an endocytic pathway.

**Bmp4 and Bmp per are reciprocally regulated through internalization**

To test if other Bmp signaling components are also internalized and/or associated with Bmp per-containing vesicles, we treated

Figure 2. Endocytic targeting of Bmp per.
(a) MECs pulsed with Bmp per conditioned media or purified Bmp per were chased with cold media or media containing chloroquine for 2 h. (b) A Western analysis for internalized Bmp per (N-terminal fragment) and activated Smad in MECs treated or pulsed with cleaved full-length recombinant Bmp per (6 nM) and harvested over 2 h (top). After pulsing with Bmp per, MECs were chased for 2 h with “cold” media lacking Bmp per (middle) or with culture medium containing 200 μM chloroquine (bottom). (c) Graphical representation of normalized intracellular Bmp per levels from the cold chase compared with the chloroquine chase from panel b.
MECs with Bmper (6 nM) and Bmp4 (0.6 nM) with or without a Bmp4 monoclonal antibody that prevents Bmp4 binding to its receptor and neutralizes Bmp4-mediated Smad phosphorylation (Fig. 3 a). If Bmp ligands and Bmp receptors participate during Bmper internalization, then their respective presence or inhibition would have an effect on endocytosis. Bmp4 alone significantly activated Smad and was weakly detected in whole cell lysates. Interestingly, Bmper and Bmp4 internalization dramatically increased in the presence of one another, whereas Bmp4-mediated Smad activation was diminished in the presence of Bmper. The Bmp4 neutralizing antibody abolished Bmper-mediated Bmp4 internalization and Smad activation, suggesting that Bmper initiates Bmp4 internalization through a receptor-dependent process. To rule out the possibility that the Bmp4 neutralizing antibody disrupts the ability of Bmper to bind Bmp4, we immunoprecipitated Bmp4 in the presence of Bmper using an antibody against Myc-Bmper, both in the absence and presence of the Bmp4 neutralizing antibody (Fig. S1 b). The neutralizing antibody did not affect the ability of Bmper to bind Bmp4. These data suggest that the neutralizing antibody and Bmper bind to Bmp4 through exclusive binding sites and that the interaction of a Bmp4–Bmper complex with Bmp receptors at the cell surface is prevented by the neutralizing antibody. Accordingly, we were able to significantly reduce the amount of internalized Bmper and Bmp4 and rescue Bmp4-mediated Smad activity by ectopically expressing a tailless form of Bmp receptor II in endothelial cells (Fig. 3 b). The mutant BmprII in this construct lacks a cytoplasmic extension downstream of the kinase domain, including sequences required for endocytosis, and emulates the naturally occurring splice variant of this receptor (Lee-Hoeflich et al., 2004). It is important to note that we were able to express these proteins via adenoviral transduction with an efficiency of ~40–50% in endothelial cells, so that the effects observed by Western blotting are an underestimate of the consequences of receptor endocytosis inhibition.

To clarify the relationships of Bmp4 and Bmper on Smad phosphorylation, we evaluated the temporal effects of Bmper on Bmp4 activity using pulse and pulse-chase experiments (Fig. 3, c–e). When treated with Bmp4 (0.6 nM) alone, Smad activation was detected for at least 24 h in MECs (Fig. 3 c, left). Attenuation of Smad phosphorylation coincided with the slow accumulation of Bmp4 in cell lysates. Bmper (6 nM) alone accumulated in MEC lysates over a 2-h interval and Smad phosphorylation was not detected (Fig. 3 c, middle). When MECs were treated with both Bmp4 (0.6 nM) and Bmper (6 nM), Bmp4 internalization was markedly enhanced and accelerated with kinetics that closely paralleled those of Bmper, which in turn internalized more efficiently in the presence of Bmp4 (Fig. 3 c, right). In the presence of Bmper, Bmp4-dependent Smad phosphorylation returned to baseline much more rapidly, within 2 h (Fig. 3 c and Fig. S1, c and d). Collectively, these data indicate that Bmper regulates Bmp4 at least in part by accelerating the removal of extracellular Bmp4.

We reasoned that, similar to Bmper, internalized Bmp4 might also be sorted for lysosomal degradation as a mechanism
to suppress its signaling. After loading exogenous proteins into cells, Bmper and Bmp4 were rapidly degraded during a chase period with cold media (Fig. 3d, top). In contrast, chasing in the presence of chloroquine prevented lysosome-dependent degradation of both Bmp4 and Bmper with similar kinetics (Fig. 3d, bottom), whereas the proteasome inhibitor MG-132 had no effect on Bmper or Bmp4 stability (not depicted). These data suggest that Bmper dampens the magnitude and duration of Bmp4-dependent Smad activation by restricting Bmp4 availability through endocytosis and raise the possibility (explored subsequently) that Bmper and Bmp4 colocalize during endosomal trafficking.

To determine whether the levels of extracellular Bmp are reduced by Bmper-mediated endocytosis, we measured the disappearance of Bmp4 from conditioned media and its accumulation within endothelial cells. Extracellular Bmp4 was efficiently removed from conditioned media within 24 h in the presence of Bmper (Fig. 3e). When MECs were retreated with Bmp4 and Bmper for 2 h before 24- and 48-h harvest time points, Bmp4 internalization remained comparable to that of acute treatment. These data indicate that the extracellular pools of Bmper and Bmp4 are being depleted concomitant with endocytosis and this process does not cause loss of the cell surface machinery (that may include recycling Bmp receptors) responsible for internalizing Bmp4 and Bmper.

Both human and zebrafish Bmper are proteolytically processed into two fragments that remain associated by disulfide bonds (Binnerts et al., 2004; Rentzsch et al., 2006). To test whether proteolytic processing of Bmper affects Bmp4 internalization and signaling activity in endothelial cell culture, we compared wild-type Bmper to three mutant forms of Bmper protein: one containing only the C-terminal fragment, another containing the N-terminal fragment, and a full-length proteolytic cleavage mutant. These myc-tagged constructs were expressed in HEK-293 cells, and the conditioned media from these cells were used to treat MECs. Wild-type Bmper behaved identically to recombinant Bmper in conditioned media from untransfected HEK-293 cells (unpublished data). Likewise the C-terminal fragment had no impact on Bmp4 signaling, whereas the N-terminal fragment and the proteolytic cleavage mutant efficiently inhibited Bmp4 signaling as measured by Smad phosphorylation (Fig. 3f). These data suggest that the N-terminal half of Bmper is required to trap Bmp4 extracellularly to prevent Smad activation through machinery that have been suggested by previous studies (Zhang et al., 2007) and that the C-terminal half of Bmper is further required to sink or internalize Bmp4 into the cell to more efficiently degrade Bmp4 and prevent signaling. Although the physiological relevance of Bmper cleavage is poorly understood, the present data suggest that Bmper proteolysis may differentially regulate Bmp activity in part by regulating its endocytosis.

**Bmper and Bmp4 internalize through a clathrin-dependent mechanism**

To explore the mechanism of Bmper internalization, we pretreated MECs with pharmacological agents that inhibit clathrin-dependent internalization into endosomes (chlorpromazine) or that disrupt lipid raft–caveolar internalization (methyl-β-cyclodextrin; Fig. 4a; Di Guglielmo et al., 2003; Hartung et al., 2006). Endosomal transport was almost completely attenuated by chlorpromazine (17 μM), whereas methyl-β-cyclodextrin (10 mM; and also the caveolin-dependent inhibitors nystatin and filipin [unpublished data]) had no effect. Collectively, this study supports a clathrin-dependent, caveolin-independent mechanism for Bmper internalization. Consistent with this conclusion, there were no differences in the ability of caveolin-1 null lung endothelial cells (Lin et al., 2007) to internalize Bmp4 in the presence of Bmper compared with their wild-type
Bmper and Bmp4 undergo endocytic trafficking to the lysosome

We used fluorescence confocal microscopy to further characterize the pathway by which Bmper and Bmp4 traffic (Fig. 5). MECs treated with Bmper and Bmp4 were first incubated at 4°C to capture the cell surface localization of Bmper. Bmp4 was located in a filamentous pattern at the cell surface, as well as in concentrated areas (possibly lipid rafts) where early endosomes begin to traffic within the cell and near filopodia (Fig. 5 a). After internalization (1-h treatment at 37°C), Bmper and Bmp4 were detected in a fraction of vesicles expressing the marker EEA1 (red), (c) The same immunofluorescence analysis was used to characterize late endocytic to lysosomal translocation using the marker Rab7 (bottom, red) to co-localize with Bmper (green) and Bmp4 (red). MECs were treated with 0.6 nM Bmp4 and 6 nM Bmper for 1.5 h before fixing. Significant colocalization of Bmper and Bmp4 overlaps with Bmper and Rab7 colocalization (right panel, yellow).
cytosis, using the well characterized Bmp inhibitor Noggin as a
(0–100 nM) on Bmp4-dependent Smad activity and Bmp4 endo-
Bmp signaling. We tested a range of Bmper concentrations
nalization would explain its opposing activities on canonical
reasoned that a threshold effect for Bmper-dependent Bmp inter-
Cv-2 stimulates Bmp signaling at low concentrations and inhibits
Bmp signaling at the cellular level and is consistent with fi ndings that
Bmper targets Bmp4 for degradation to restrict its
Bmp4 – Bmper complex. We also found that Noggin triggered
Bmp4 with a 2:1 molar stoichiometry (Zhang et al., 2007), and
remarkably it was when this molar ratio was exceeded that
Bmp4, Bmper enhanced Smad activation, whereas at molar con-
centrations higher than Bmp4, Bmper attenuated Bmp4-mediated
endocytosis at concentrations that exceeded stoichiomet-
ric equivalence with Bmp4 (Fig. 6 a). The kinetics of Bmper- and
Bmp4 activity. The N-terminal fragment of
control (Fig. 6, a and b). At molar concentrations below those of
Bmp4, Bmper enhanced Smad activation, whereas at molar con-
centrations higher than Bmp4, Bmper attenuated Bmp4-mediated
Smad activation and simultaneously triggered Bmp4 endocytosis
(Fig. 6, a and b). Binding assays indicate that Bmper binds to
Bmp2 with a 2:1 molar stoichiometry (Zhang et al., 2007), and
remarkably it was when this molar ratio was exceeded that
Bmp4 signaling and triggered endocytosis of the
Bmp4–Bmper complex. We also found that Noggin triggered
Bmp4 endocytosis at concentrations that exceeded stoichiometric
 equivalence with Bmp4 (Fig. 6 a). The kinetics of Bmper- and
Noggin-dependent Bmp4 internalization were similar, yet only
Bmper increased Bmp4-dependent Smad signaling at below
threshold concentrations. The selectivity of this endocytic mecha-
nism for other secreted modulators of Bmp signaling is further
demonstrated in the results describing Fig. 8.

We explored the concentration-dependent relationship of
Bmp4 activity in a concentration-dependent manner.

Our discovery of a regulatory role for Bmper to regulate Bmp
signaling within the endocytic pathway provides an elegant
explanation for the inhibitory effects of Bmper on Bmp activity
identified by multiple laboratories (Moser et al., 2003; Binnerts
et al., 2004; Coles et al., 2004; Rentzsch et al., 2006; Zhang et al.,
2007), but fails to reconcile other studies that indicate pro-Bmp
activities of Bmper (Conley et al., 2000; Coles et al., 2004;
Kamimura et al., 2004; Ikeya et al., 2006; Rentzsch et al., 2006).
A recently reported concentration-dependent model helps
explain both anti- and pro-Bmp activity by showing that Drosophila
Cv-2 stimulates Bmp signaling at low concentrations and inhibits
Bmp signaling at high concentrations (Serpe et al., 2008). We
reasoned that a threshold effect for Bmper-dependent Bmp inter-
nalization would explain its opposing activities on canonical
Bmp signaling. We tested a range of Bmper concentrations
(0–100 nM) on Bmp4-dependent Smad activity and Bmp4 endo-
cytosis, using the well characterized Bmp inhibitor Noggin as a
collective.
specify defects during embryonic development. Given the important role of Bmp4 signaling in lung development and our findings that Bmp4 and Bmper share a concentration-dependent relationship in the regulation of lung MEF survival we were particularly interested in examining the structural defects in the lungs of Bmper-deficient mice. In the perinatal lung of Bmper +/−/H11002 mice, an overgrowth of mesenchymal cells (which normally express Bmper) appeared to prevent complete alveoli expansion, as indicated by smaller and fewer terminal sacs that were separated by thickened interstitial mesenchyme (Fig. 7a). These anatomical defects are similar to lung phenotypes reported previously in Bmper-deficient mice (Ikeya et al., 2006). Upon closer examination of the cellular and molecular features resulting from a decrease in Bmper expression, we detected several anomalies that were consistent with a "pro-Bmp" effect in the Bmper +/− mice, an overgrowth of mesenchymal cells (which normally express Bmper) appeared to prevent complete alveoli expansion, as indicated by smaller and fewer terminal sacs that were separated by thickened interstitial mesenchyme (Fig. 7a). These anatomical defects are similar to lung phenotypes reported previously in Bmper-deficient mice (Ikeya et al., 2006). Upon closer examination of the cellular and molecular features resulting from a decrease in Bmper expression, we detected several anomalies that were consistent with a "pro-Bmp" effect in the Bmper +/− mice, an overgrowth of mesenchymal cells (which normally express Bmper) appeared to prevent complete alveoli expansion, as indicated by smaller and fewer terminal sacs that were separated by thickened interstitial mesenchyme (Fig. 7a). These anatomical defects are similar to lung phenotypes reported previously in Bmper-deficient mice (Ikeya et al., 2006). Upon closer examination of the cellular and molecular features resulting from a decrease in Bmper expression, we detected several anomalies that were consistent with a "pro-Bmp" effect in the Bmper +/− mice, an overgrowth of mesenchymal cells (which normally express Bmper) appeared to prevent complete alveoli expansion, as indicated by smaller and fewer terminal sacs that were separated by thickened interstitial mesenchyme (Fig. 7a). These anatomical defects are similar to lung phenotypes reported previously in Bmper-deficient mice (Ikeya et al., 2006).
is deficient to make way for infiltrating epithelial cells that go on to form the terminal buds (Weaver et al. 1999). It is also a region where both Bmpr and Bmp4 are normally expressed (Bellusci et al. 1996 and Fig. S3), suggesting that without Bmpr, Bmp4 activity is allowed to signal unabated, thereby reducing the amount of mesenchymal apoptosis, a theory that is supported by our in vitro measurements of proapoptotic activity of Bmpr in explanted embryonic lung mesenchymal cells (Fig. 6, c and d).

To confirm at the molecular level that the observed changes in Bmpr +/− lung tissue were a result of increased Bmp signaling, we measured the amount of Id1, a downstream Bmp target (Hollnagel et al., 1999), in lung tissue of both Bmpr +/− and wild-type mice by PCR (Fig. 7 c). As expected, there was an appreciable increase in Id1 expression in Bmpr +/− lungs, further indicating that the reduction in Bmpr expression leads to a pro-Bmp effect in this tissue. These data suggest that the fate of the lungs to mature in Bmpr +/− mice is caused by sustained Bmp4 signaling in the distal region of the lung (resulting in a failure of the mesenchyme to recede) and not by a nonspecific secondary effect of delayed lung development. Collectively, these data support a tissue-specific and physiologically relevant anti-Bmp role for Bmpr and are also consistent with the anti-Noggin but not Chordin (Serpe et al., 2008).

If increased Bmp activity in Bmpr +/− mice is at least partially caused by impairment of Bmpr-mediated Bmp4 endocytosis, then one would anticipate decreased cytoplasmic accumulation of Bmp4 in the cytosol of lung cells. In isolated cell preparations from mouse lung stripped of membrane, Bmp4 was found to accumulate in the cytosol of wild-type lung cells in substantial levels, but was present in markedly reduced levels in both Bmpr +/− and −/− lungs (Fig. 7 d), indicating that Bmpr is necessary for optimal internalization of Bmp in vivo, as it is in vitro. These observations, combined with the aforementioned finding of increased Bmp signaling in Bmpr +/− lung tissue and our in vitro studies, indicate strongly that Bmpr exerts a physiologically relevant anti-Bmp effect and supports a mechanistic model whereby BMPER regulates Bmp signaling in part by promoting Bmpr endocytosis and subsequent lysosomal degradation.

Endocytosis mediated by Gremlin and Noggin but not Chordin

Discrete but overlapping developmental expression patterns of multiple Bmp regulators during patterning (Srinivasan et al., 2002; Ralston and Blair, 2005; Rentzsch et al., 2006) suggest mutually dependent interactions among these factors. The specificity of Bmpr-mediated endocytosis of Bmp4 prompted us to test the effects of recombinant Chordin, Noggin, and Gremlin on Bmp4-mediated Smad activation and endocytosis to determine the extent to which this novel endocytic mechanism extends to other classical extracellular regulators of Bmp signaling. Under all conditions tested, Bmpr induced Bmp4 internalization while reducing Bmp4-mediated Smad activity to a level intermediate to that of Bmp4-treated and untreated cells (Fig. 8, a and b). Consistent with previous reports of their potent Bmp inhibitory activity, Noggin and Gremlin eliminated Bmp4-mediated Smad activation, irrespective of Bmpr treatment, and led to Bmp4 endocytosis (Fig. 8 b, middle and bottom). The apparent affinities of Noggin and Gremlin for Bmp4 are higher than Bmpr, as they abolished Bmpr internalization and more potently reduced Bmp4-mediated Smad phosphorylation. The ability of Noggin and Gremlin to induce Bmp4 internalization suggests that endocytic internalization of Bmps also accounts, at least in part, for their ability to inhibit Bmp activity. In contrast, Chordin had no effect on Bmp4-induced Smad activation or endocytosis. Although Chordin was inactive in the presence of Bmp4 alone, Bmpr induced Chordin internalization. Interestingly, internalization of Bmp4, Bmpr, and Chordin were attenuated when all of these factors were present simultaneously (Fig. 8 c), suggesting that higher order protein complexes may actually suppress the endocytic mechanism.

We conducted further studies to determine whether Noggin also targets Bmp4 for the lysosome and whether Bmpr has this effect on Chordin (Fig. 8 c). The effects of Noggin on Bmp4 were similar to the effects of Bmpr on Bmp4 (Fig. 8 c, middle), as Bmp4 was degraded during the cold chase, but not in the presence of chloroquine. The proteolytic processing of Bmp4 in the presence of Noggin was not as thorough as when Bmpr was in the presence of Bmp4, suggesting there may be subtle differences in Bmp proteolysis and possibly the kinetics of elimination elicited by different Bmp modulators. Interestingly, Noggin was constitutively internalized by the endothelial cells. This is in contrast to Chordin, which was not internalized in the presence of Bmp4, whether or not Bmpr was present. However, in the presence of Bmpr alone under these culture conditions (Fig. 8, b and c), Chordin was internalized and eliminated after a cold chase. Interestingly, intracellular Chordin did not accumulate after chloroquine chase. It is possible that internalized Chordin is eliminated by the cell independent of the lysosome (i.e., via proteasomal degradation) or that the kinetics of its turnover differs from Noggin and Bmpr. That the intracellular trafficking of Chordin is distinct from Bmpr and Bmp modulators such as Noggin and Gremlin may reflect the higher affinity of Bmpr for a Bmp4-Chordin complex than it does for Chordin alone. Collectively, these data suggest a complex and competitive interplay among Bmp modulators for Bmps and underscore a generalized role for activities within the endocytic compartments among selective Bmp modulators.

Discussion

Several models have been proposed to explain the anti- and pro-Bmp mechanisms of Bmpr (Coles et al., 2004). Recently, a paper using biochemical and genetic studies in Drosophila proposes a model where Cv-2 can enhance and inhibit Bmp signaling at low and high concentrations, respectively (Serpe et al., 2008). Dependency on concentration and proteolytic cleavage have also been reported for other extracellular modulators of Bmp activity (Larrain et al., 2001). Our data provide an additional mechanism whereby proteolysis and concentration dependency fine-tune Bmp signaling through interactions with secreted proteins.

Although both proteolytic activation and inactivation have been described for several Bmp factors, the degradation of secreted Bmps is incompletely understood. To date, limited data...
participates in creating an inverse gradient of the Bmp modulator Sog (a Chordin homologue), which fine-tunes concentration gradients for Dpp in the extracellular space (Srinivasan et al., 2002). Receptors for Bmp family members have also been shown to internalize through distinct endocytic pathways. In the case of TGF-β/H9252 receptors, caveolin-dependent internalization mechanisms target the receptors for proteasomal degradation, whereas clathrin-dependent internalization alternatively is required for appropriate Smad activation (Di Guglielmo et al., 2003). Constitutive internalization and degradation of type I and II Bmp receptors via clathrin-mediated endocytosis, and to a lesser extent internalization of BmprII by a caveolae-dependent route, have also been established (Hartung et al., 2006). Our experimental observations extend the role of endocytic mechanisms within the Bmp signaling pathway by demonstrating mutually triggered clathrin-dependent endocytosis are available describing Bmp degradation (Entchev et al., 2000; Degnin et al., 2004). Lysosomal- and proteasomal-dependent degradation of Bmp4 within the presecretory pathway was shown to occur after the initial cleavage step of the inactive Bmp4 precursor and before the final cleavage step that stabilizes the mature secreted form (Degnin et al., 2004). Postsecretory regulation of Dpp through an endocytic mechanism that traffics DPP to the lysosome for degradation during Drosophila development has also been described. The present study provides the first example of targeted intracellular Bmp degradation by a secreted regulator of Bmp signaling and indicates that this endocytic mechanism is broadly used by Bmp regulators such as Bmper, Noggin, and Gremlin to fine-tune Bmp regulation.

Indications that endocytic internalization mechanisms modulate Bmp signaling were first suggested by studies in Drosophila showing that a Dynamin-dependent retrieval process participates in creating an inverse gradient of the Bmp modulator Sog (a Chordin homologue), which fine-tunes concentration gradients for Dpp in the extracellular space (Srinivasan et al., 2002). Receptors for Bmp family members have also been shown to internalize through distinct endocytic pathways. In the case of TGF-β receptors, caveolin-dependent internalization mechanisms target the receptors for proteasomal degradation, whereas clathrin-dependent internalization alternatively is required for appropriate Smad activation (Di Guglielmo et al., 2003). Constitutive internalization and degradation of type I and II Bmp receptors via clathrin-mediated endocytosis, and to a lesser extent internalization of BmprII by a caveolae-dependent route, have also been established (Hartung et al., 2006). Our experimental observations extend the role of endocytic mechanisms within the Bmp signaling pathway by demonstrating mutually triggered clathrin-dependent endocytosis

Figure 8. Bmp4 endocytosis is mediated by Bmper, Gremlin, and Noggin, but not Chordin. A panel of secreted Bmp factors (6.0 nM) were evaluated for their effects on Bmp4 (0.6 nM) and on Bmper-mediated Bmp4 internalization and signaling at several time points. A Western analysis showing the effects of active recombinant Chordin, Noggin, and Gremlin on the activity of Bmp4 alone or with cleaved full-length Bmper was measured 10 min (a) and 1.5 h (b) after treatment. Protein internalization and Smad activation were measured for each of these Bmp factors: Chordin (top), Noggin (middle), and Gremlin (bottom). (c) Pulse-chase experiment in MECs treated with Bmp4, Bmper, Noggin, and combinations of Bmp4 and Bmper (top), Bmp4 and Noggin (middle), and Bmper and Chordin (bottom). After treating MECs with recombinant protein(s) using the same experimental conditions and protein concentrations used in panel a, MECs were either chased with cold media or cold media containing chloroquine that lack recombinant protein(s). (d) A model of Bmper-mediated Bmp4 internalization.
of Bmpr and Bmp4 and significant involvement of surface Bmp receptors in mediating endocytic sorting of Bmpr and Bmp4 to the lysosome to limit the temporal boundaries of Smad activation.

The inhibitory activity of Bmpr is supported by our in vivo evidence that Bmpr behaves as a tissue-specific Bmp inhibitor in the developing lung of Bmpr-deficient mice. The deletion of Bmpr affects the distal area of the lung where Bmpr is expressed in the mesenchyme, but not the proximal area where both Calcitonin gene-related peptide and Clara cells are located. These results strongly support the interpretation that the phenotype in Bmpr +/− lungs is caused by local deficiency of Bmpr and not by a nonspecific secondary effect of delayed lung development. The physiological anti-Bmp activity of Bmpr during lung development is completely consistent with our in vitro evidence that Bmpr leads to apoptosis of embryonic interstitial lung MEFs, which is normally inhibited by Bmp signaling. Whether Bmpr exerts pro- or anti-Bmp activities in vivo in different models may be tissue and possibly species specific and tissue differences in the ratios of Bmpr to Bmp family members likely determine whether Bmpr is augmenting or suppressing Bmp signaling.

Collectively with previous biochemical and genetic studies (Coles et al., 2004; Rentzsch et al., 2006; Zhang et al., 2007; Serpe et al., 2008), a model can be developed that parsimoniously accounts for seemingly disparate observations (Fig. 8 d). At low molar concentrations, Bmpr may bind Bmp4 in a manner that increases the affinity of Bmp4 for its receptors (Zhang et al., 2007), accounting for the activation of Bmp signaling that we observe under these conditions (Fig. 6 a). A recent study suggests that this occurs through a Bmp, Bmpr, and Bmp receptor tripartite complex that facilitates transfer of Bmp to the Bmpr receptor to account for this activity (Serpe et al., 2008). As Bmpr exceeds the molar concentration of Bmp4, high affinity Bmp4 receptor binding determinants may be masked, resulting in a low affinity receptor interaction, but in any event endocytosis of both Bmp4 and Bmpr is clearly triggered under these circumstances in a receptor-dependent fashion. The uncleaved form of Bmpr was previously suggested to exert its anti-Bmp activity through association with components of the extracellular matrix and cell membrane (Rentzsch et al., 2006), but the present studies make clear that the uncleaved form of Bmpr also enhances Bmp4 endocytosis, facilitating a Bmp4 sink through recruitment of Bmps to the cell surface. Furthermore, although the N terminus of Bmpr binds Bmp4, the C terminus of Bmpr is required for this endocytic mechanism; our results and those of other groups lead us to speculate that the C terminus of Bmpr may facilitate endocytosis through interactions with the extracellular matrix and/or Bmp receptors. Under certain conditions, the function of trapping Bmps by Bmpr can be uncoupled from an intracellular Bmp sink, and therefore endocytosis is not essential to the inhibitory activity of Bmpr. The ability of not only Bmpr but also Gremlin and Noggin to trigger the endocytosis of Bmp4 suggests that this is a broadly applied mechanism by inhibitors of Bmp signaling to regulate the concentration and temporal availability of Bmps as a signal regulation strategy.

Materials and methods

Antibodies and reagents

The antibodies and recombinant proteins for Bmpr, Bmp4, Noggin, Chordin, and Gremlin and monoclonal anti-human Bmp4 neutralizing antibody were purchased from R&D Systems. Phosphorylated Smad and cleaved Caspase 3 antibodies were purchased from Cell Signaling Technology. GuikChange site-directed mutagenesis (Agilent Technologies) was used to modify a previously described pSecTag2 vector containing wild-type Bmpr (Moser et al., 2003; W.C. Sessa [Yale University School of Medicine, New Haven, CT] provided the lung endothelial cells null for caveolin-1.

Preembedding immunoelectron microscopy

MECs were fixed with 4% paraformaldehyde/0.5% glutaraldehyde. After three washes in 0.15 M sodium phosphate buffer the cells were immunostained during a preembedding immunogold-silver procedure developed by Yi et al. (2001). 70nm ultrathin sections were cut using a diamond knife and an Ultracut UCT microtome (Leica), mounted on 200 mesh copper grids and post-stained with Reynolds’ lead citrate for 8 min. Sections were examined on a transmission electron microscope (EM-910; Carl Zeiss, Inc.) using an acceleration voltage of 80 kV and a 75-μm objective aperture. Digital micrographs were taken using an Orion digital camera (Gatan, Inc.).

Cell culture

MECs were isolated from mouse lung at embryonic day 15.5 from wild-type mice of a mixed background (C57BL/6 and Ola 129). In brief, embryos were dissected from the mother’s uterus and placed in sterile PBS in a 10-cm dish. The embryos were separated from fetal membranes (in the case of lung MEFs, lung lobes were separated from the heart and neighboring connective tissue) and placed in 1 ml of 0.25% trypsin-EDTA and a sterile razor blade was used to mince the embryos. The minced embryos were allowed to sit in the trypsin-EDTA for 10 min at 37°C and 5% CO2 followed by neutralization in 2 ml of growth media [DME, 10% FCS, and penicillin and streptomycin with 50 μM β-mercaptoethanol]. After repeatedly pipetting and disaggregating the tissue, 8 ml of growth media was added to the plates to seed the fibroblasts. On day 2, the 10-cm plates were confluent and cells in suspension were removed. The cells were passed once and then used for experimentation. MECs were cultured as previously described (Moser et al., 2003; Ren et al., 2007) and human coronary arterial endothelial cells (HCAECs; Lonza) were cultured according to the manufacturer’s recommendation using EGM-2 media (Lonza).

Unless otherwise indicated, cultured cells were treated with recombinant Bmp4 (0.6 nM) and/or recombinant Bmpr, Chordin, Noggin, and/or Gremlin (6.0 nM) in high glucose DME for various times to analyze the temporal effects of Bmpr toward Bmp4 activity and internalization. For chase experiments, cells were pulsed with recombinant proteins and then chased with “cold” Bmp and DNase. Cells were chased with chloroquine for the indicated times. Active internalization was determined in cells cultured normally at 37°C versus pretreating the cells at 4°C for 45 min before treating them with recombinant proteins for up to 60 min at 4°C. Conditioned media were harvested after 48 h of transfecting HEK-293 cells using FuGENE and CDNA from wild-type Bmpr or Bmpr mutants. Undiluted condition media were applied to MECs, and recombinant proteins used in the mutant studies were diluted in control (untransfected) HEK-293 condition media as positive controls for internalization and Bmp signaling.

Subcellular localization

Immunolocalization was performed on untreated and treated MECs and HCAECs, fixed with 4% paraformaldehyde, permeabilized with 0.1% Triton X-100, blocked with 1% BSA in PBS, and incubated with primary antibodies, followed by incubation with Alexa Fluor secondary antibodies. Digital pictures were taken using an upright laser scanning confocal microscope (SP2 AOBBS; Leica) at room temperature (at the University of North Carolina’s Michael Hooker Microscopy Facility). Images were processed using LCS Lite imaging software (Leica) and Photoshop (Adobe).

Western blotting analysis

Western analysis was performed on MECs treated with recombinant proteins (all purchased from R&D Systems). In brief, blotting was performed by incubating primary antibodies (anti-Bmpr [R&D Systems], anti-Bmp4 [Millipore], anti-Chordin, anti-Gremlin, and anti-Noggin [R&D Systems]). Blots were developed with an Advanced ECL kit (Thermo Fisher Scientific). Immunoprecipitation was performed using an anti-Myc antibody (Santa Cruz Biotechnology, Inc.)
RNA isolation and real-time PCR

Total RNA was extracted from cells and tissues using RNeasy kits according to the manufacturer’s instructions (QIAGEN). First-stand cDNA was synthesized using 500 ng of total RNA with 200 U of Superscript II RNase H-T (Invitrogen) in a final volume of 20 μl. The resulting products were then treated with RNase A for 30 min at 37°C and purified thereafter with Qiaquick PCR purification kit (QIAGEN). Real-time PCR was performed using the 7500 Real-time PCR system (Applied Biosystems).

Lung histology

Mouse tissues were fixed overnight in 4% paraformaldehyde/PBS and processed for paraffin embedding. Deparaffinization sections (5 μm) were treated with Antigen unmasking reagent (Vector Laboratories) for 15 min for antigen retrieval. Blocking was achieved with 2.5% horse serum in PBS for 10 min. Primary antibody (anti-GFP and anti–prosurfactant C) incubations were performed for 30 min at room temperature. Biotinylated secondary antibodies (Vector Laboratories) were added to sections for 10 min, followed by signal detection using NovaRED reagent (Vector Laboratories).

Anti–prosurfactant C antibody was a gift from J. Whitsett (Cincinnati Children’s Hospital Medical Center, Cincinnati, OH).

TUNEL assay

TUNEL assay was performed according to the manufacturer’s instructions (Promega). In brief, paraformaldehyde-fixed and paraffin-embedding lung tissue sections were stained with fluorescein-12-dUTP (Promega). Images were acquired using a confocal microscope (Carl Zeiss, Inc.).

Target deletion of mouse BMPER gene

Mouse deficient for BmpER were generated with standard gene targeting methods (Dai et al., 2003). The targeting construct was generated using pOSfrt (Caron and Smithies, 2001) as the backbone, which contains (a) a 4.4-kb PCR-generated fragment from genomic DNA that includes the BMPER promoter; (b) a 700-bp cDNA encoding EGFP (Clontech Laboratory Inc.); (c) a 300-bp bovine growth hormone poly(A) addition region; and (d) a promoter; (b) a 700-bp cDNA encoding EGFP (Clontech Laboratory Inc.); and (d) a 2.0 kb PCR-generated genomic fragment from second intron of the BMPER gene. Embryonic stem (ES) cells (129/ola) were electroporated with the targeting construct. ES colonies that were G418/ganciclovir resistant were identified. ES colonies were injected into C57BL/6 blastocysts (Dai et al., 2003). Male chimeras were mated to wild-type C57BL/6 females to establish an isogenic line, and all experiments were conducted on the resulting hybrid background. The C57BL/6J 129 ES cells null for caveolin-1. We also thank Andrea Portbury for her critical review of the manuscript.

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