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

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Bmper, 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 Bmper, although the molecular mechanisms through which Bmper affects Bmp signaling are unclear. In this paper, we demonstrate that as molar concentrations of Bmper exceed Bmp4, Bmper 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 Bmper and Bmp4 by the lysosome. Bmper-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 Bmper 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 Bmper (Bmp-binding endothelial cell precursor-derived regulator), a member of the Kielin-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 Bmper serving both as a pro- and anti-Bmp factor failed to support a model that explained the dual nature of Bmper 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 Bmper 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 Bmper behaves in a concentration-dependent manner. Bmper enhances Bmp4-mediated Smad activation at molar concentrations below that of Bmp4 in endothelial cells. Alternatively, when Bmper 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

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Abbreviations used in this paper: ES, embryonic stem; HCAEC, human coronary arterial endothelial cell; MEC, mouse endothelial cell; MEF, mouse embryonic fibroblast.

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Supplemental material can be found at: http://doi.org/10.1083/jcb.200808064
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 that this may be a broadly applied but selective mechanism for regulating Bmp signaling.

**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 this 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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At 37°C, Bmp per internalization (as detected by its presence in cell lysates) steadily increased over time. However, Bmp per internalization was barely detected at 4°C, and the small amount of Bmp per detected after 20 min of treatment at 4°C was nearly eliminated by MECs after 1 h of treatment. The disappearance of both the N and C terminus of Bmp per over the time course of this experiment suggest that Bmp per is not only internalized but may also be further processed or degraded intracellularly. To distinguish internalized Bmp per and Bmp4 from their accumulation at the cell surface, we stripped the plasma membrane with an acid wash procedure after a pulse-chase experiment (Fig. S1 a, available at http://www.jcb.org/cgi/content/full/jcb.200808064/DC1). This procedure had no effect on the detection of the internalized Bmp proteins in whole cell lysates and confirmed that the accumulation of Bmp per in cell lysates is a result of endocytosis and not caused by increased cell surface affiliation. Bmp per internalization was detected in endothelial cells, rat neonatal ventricular cardiomyocytes, and mouse embryonic fibroblasts (MEFs), but not by HEK-293 or COS7 cells (Fig. 1 b), indicating that the machinery required for Bmp per internalization is restricted to a limited range of cell types.

We used fluorescence confocal microscopy to confirm the localization of Bmp per in both untreated MECs and MECs treated with recombinant Bmp per (Fig. 1, c–h). In untreated MECs, endogenous Bmp per was detected at low levels with a distinct punctate perinuclear pattern. This more sensitive immunofluorescent staining likely represents accumulation of a low abundance of protein within the secretory pathway, as this form of Bmp per was detectable at extremely low levels when using the same antibody for Western analysis. In contrast, a significant amount of Bmp per accumulated near the plasma membrane after treating the MECs for 5 min with recombinant Bmp per (Fig. 1 g). When MECs were chased with cold media (lacking recombinant protein), levels of intracellular Bmp per returned to their endogenous levels (unpublished data), suggesting that internalized Bmp per undergoes efficient degradation. When MECs treated with Bmp per were chased with cold media containing the lysotropic agent chloroquine (Fig. 1 h), Bmp per accumulated in large punctate vesicles that were clearly distinct in pattern and abundance from that seen with endogenous Bmp per within the secretory pathway or after a 5-min pulse (Fig. 1 i).

To explore the possibility that internalized Bmp per undergoes lysosome-dependent degradation, as suggested by the effects of chloroquine in our immunofluorescence experiments, we next compared the endocytic behavior of purified recombinant Bmp per to Bmp per secreted in conditioned media from HEK-293 cells transiently transfected with a plasmid encoding full-length Bmp per. The HEK-293 conditioned media contained both full-length and “cleaved” Bmp per, indicating that the secretory compartment of these cells is capable of cleaving the propeptide of Bmp per through intracellular pH-dependent catalysis (Thuveson and Fries, 2000; Ambrosio et al., 2008; Serpe et al., 2008) or that these cells might express a yet to be identified protease capable of cleaving Bmp per. The recombinant Bmp per used in these studies is nearly completely cleaved. MECs were pulsed with either of the two sources of Bmp per 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. 2 a). 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. 2, b 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...
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 Bmp receptor on Smad phosphorylation, we evaluated the temporal effects of Bmp receptor 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. Bmp receptor (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 Bmp receptor (6 nM), Bmp4 internalization was markedly enhanced and accelerated with kinetics that closely paralleled those of Bmp receptor, which in turn internalized more efficiently in the presence of Bmp4 (Fig. 3 c, right). In the presence of Bmp receptor, Bmp4-dependent Smad activation was diminished in the presence of Bmp receptor. The Bmp4 neutralizing antibody abolished Bmp receptor-mediated Bmp4 internalization and Smad activation, suggesting that Bmp receptor initiates Bmp4 internalization through a receptor-dependent process. To rule out the possibility that the Bmp4 neutralizing antibody disrupts the ability of Bmp4 to bind Bmp receptor, we immunoprecipitated Bmp4 in the presence of Bmp receptor using an antibody against Myc-Bmp receptor, both in the absence and presence of the Bmp4 neutralizing antibody (Fig. S1 b). The neutralizing antibody did not affect the ability of Bmp receptor to bind Bmp4. These data suggest that the neutralizing antibody and Bmp receptor bind to Bmp4 through exclusive binding sites and that the interaction of a Bmp4–Bmp receptor complex with Bmp receptor at the cell surface is prevented by the neutralizing antibody. Accordingly, we were able to significantly reduce the amount of internalized Bmp receptor 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.

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We reasoned that, similar to Bmp receptor, internalized Bmp4 might also be sorted for lysosomal degradation as a mechanism
cells, and the conditioned media from these cells were used to treat MECs. Wild-type Bmpr behaved identically to recombinant Bmpr 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 Bmpr 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 Bmpr is further required to sink or internalize Bmp4 into the cell to more efficiently degrade Bmp4 and prevent signaling. Although the physiological relevance of Bmpr cleavage is poorly understood, the present data suggest that Bmpr proteolysis may differentially regulate Bmp activity in part by regulating its endocytosis.

Bmpr and Bmp4 internalize through a clathrin-dependent mechanism

To explore the mechanism of Bmpr internalization, we pre-treated 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 Bmpr 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 Bmpr compared with their wild-type cells, and the conditioned media from these cells were used to treat MECs. Wild-type Bmpr behaved identically to recombinant Bmpr 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 Bmpr 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 Bmpr is further required to sink or internalize Bmp4 into the cell to more efficiently degrade Bmp4 and prevent signaling. Although the physiological relevance of Bmpr cleavage is poorly understood, the present data suggest that Bmpr proteolysis may differentially regulate Bmp activity in part by regulating its endocytosis.

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Bmp4 and Bmp4 undergo endocytic trafficking to the lysosome

We used fluorescence confocal microscopy to further characterize the pathway by which Bmp4 and Bmp4 traffic (Fig. 5). MECs treated with Bmp4 and Bmp4 were first incubated at 4°C to capture the cell surface localization of Bmp4. Bmp4 was located near a caveolin compartment at the cell surface and internalizes into early endosomes (Fig. 4 b) using 25-nm immunoparticles to detect Bmp4 and 5-nm particles to detect Bmp4. At early time points, Bmp4 and Bmp4 congregated along the cell membrane (Fig. 4 b, i), presumably as they associate together with Bmp receptors. The Bmp4–Bmp4 heterodimers soon coalesced over a dense membrane that is characteristic of clathrin-dependent invaginations (Fig. 4 b, ii and iii). These clathrin-coated pits were subsequently visualized within the cytoplasm (Fig. 4 b, iv), and transfer of these components to mature endosomal compartments was apparent (Fig. 4 b, v; arrow). In the absence of Bmp4, Bmp4 was not detected intracellularly by electron microscopy after 1.5 h of treatment. Collectively, these data are consistent with a clathrin-dependent mechanism for internalizing both Bmp4 and Bmp4, which is consistent with recent papers describing clathrin-dependent internalization of TGF-β and Bmp receptors (Di Guglielmo et al., 2003; Hartung et al., 2006).
further during trafficking using Rab7, a GTPase that marks the late endosome compartment (Bucci et al., 2000; Fig. 5 c). Even at this late stage in vesicular transport, Bmpr and Bmp4 remained intimately colocalized within perinuclear Rab7-expressing endosomes. The physical proximity and functional role of Rab7 in lysosomal degradation provides an additional layer of evidence that Bmpr targets Bmp4 for degradation to restrict its signaling at the cellular level and is consistent with findings that a Rab7-dependent endocytic transport mechanism targets Dpp for degradation to establish a long range Dpp concentration gradient during *Drosophila* development (Entchev et al., 2000).

**Bmpr regulates Bmp4 activity in a concentration-dependent manner**

Our discovery of a regulatory role for Bmpr to regulate Bmp signaling within the endocytic pathway provides an elegant explanation for the inhibitory effects of Bmpr 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 Bmpr (Conley et al., 2000; Coles et al., 2004; 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 Bmpr-dependent Bmp internalization would explain its opposing activities on canonical Bmp signaling. We tested a range of Bmpr concentrations (0–100 nM) on Bmp4-dependent Smad activity and Bmp4 endocytosis, using the well characterized Bmp inhibitor Noggin as a control (Fig. 6, a and b). At molar concentrations below those of Bmp4, Bmpr enhanced Smad activation, whereas at molar concentrations higher than Bmp4, Bmpr attenuated Bmp4-mediated Smad activation and simultaneously triggered Bmp4 endocytosis (Fig. 6, a and b). Binding assays indicate that Bmpr binds to Bmp2 with a 2:1 molar stoichiometry (Zhang et al., 2007), and remarkably it was when this molar ratio was exceeded that Bmpr restricted Bmp4 signaling and triggered endocytosis of the Bmp4-Bmpr complex. We also found that Noggin triggered Bmp4 endocytosis at concentrations that exceeded stoichiometric equivalence with Bmp4 (Fig. 6 a). The kinetics of Bmpr- and Noggin-dependent Bmp4 internalization were similar, yet only Bmpr increased Bmp4-dependent Smad signaling at below threshold concentrations. The selectivity of this endocytic mechanism for other secreted modulators of Bmp signaling is further demonstrated in the results describing Fig. 8.

We explored the concentration-dependent relationship of Bmpr with Bmp4 in a biologically relevant model of Bmp signaling. At day 15.5 of embryonic lung development, the lung mesenchyme begins to regress through a Bmp4-dependent mechanism as the terminal epithelium expands into the distal airways (Belluscì et al., 1996). We found Bmpr to be expressed in the supporting mesenchyme of the lung (Fig. S3, available at http://www.jcb.org/cgi/content/full/jcb.200808064/DC1), which is consistent with a previous study (Ikeya et al., 2006). Bmp4, in contrast, is expressed in the distal tips of the lung epithelium and at lower levels in the adjacent lung mesenchyme (Belluscì et al., 1996). If a concentration-dependent model for regulating Bmp signaling by Bmpr is correct, then Bmpr should also regulate Bmp4-mediated survival of the embryonic lung mesenchyme in a concentration-dependent manner. To test this theory, we treated
specific defects during embryonic development. Given the important role of Bmp4 signaling in lung development and our findings that Bmp4 and BmpR 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 BmpR-deficient mice. In the perinatal lung of BmpR+/H11002 mice, an overgrowth of mesenchymal cells (which normally express BmpR) 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 BmpR-deficient mice (Ikeya et al., 2006).

Upon closer examination of the cellular and molecular features resulting from a decrease in BmpR expression, we detected several anomalies that were consistent with a “pro-Bmp” effect in the BmpR+/- lungs. At embryonic day 18.5, there was an increased ratio of type II epithelial cells to total distal cells (Fig. 6c) as well as fewer apoptotic cells detected by TUNEL assay in BmpR+/- lungs compared with wild-type mice (not depicted). This abundance of interstitial mesenchymal tissue was not caused by altered differentiation or increased cell proliferation as neither of these parameters were significantly altered in BmpR+/- lungs compared with wild-type littermates. Not surprisingly, this observed phenotype was heavily localized to the distal regions of the developing lung, the region in which lung mesenchyme usually regresses if Bmp signaling...
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 Bmper and Bmp4 are normally expressed (Belluscio et al. 1996 and Fig. S3), suggesting that without Bmper, 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 Bmper in explanted embryonic lung mesenchymal cells (Fig. 6, c and d).

To confirm at the molecular level that the observed changes in Bmper +/- 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 Bmper +/- and wild-type mice by PCR (Fig. 7 c). As expected, there was an appreciable increase in Id1 expression in Bmper +/- lungs, further indicating that the reduction in Bmper expression leads to a pro-Bmp effect in this tissue. These data suggest that the fate of the lungs to mature in Bmper +/- 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 Bmper and are also consistent with the anti-Bmp effects of Bmper in the developing pupal wing of Drosophila (Serpe et al., 2008).

If increased Bmp activity in Bmper +/- mice is at least partially caused by impairment of Bmper-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 Bmper +/- and -/- lungs (Fig. 7 d), indicating that Bmper 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 Bmper +/- lung tissue and our in vitro studies, indicate strongly that Bmper exerts a physiologically relevant anti-Bmp effect and supports a mechanistic model whereby BMPER regulates Bmp signaling in part by promoting Bmp 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 Bmper-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, Bmper 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 Bmper treatment, and led to Bmp4 endocytosis (Fig. 8 b, middle and bottom). The apparent affinities of Noggin and Gremlin for Bmp4 are higher than Bmper, as they abolished Bmper internalization and more potently reduced Bmp4-mediated Smad phosphorylation. The ability of Noggin and Gremlin to induce Bmp4 internalization suggests that endocytic internalization of Bmp4 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, Bmper induced Chordin internalization. Interestingly, internalization of Bmp4, Bmper, 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 Bmper has this effect on Chordin (Fig. 8 c). The effects of Noggin on Bmp4 were similar to the effects of Bmper 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 Bmp4 was in the presence of Bmper, 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 Bmper was present. However, in the presence of Bmper 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. 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 Bmper. That the intracellular trafficking of Chordin is distinct from Bmper and Bmp modulators such as Noggin and Gremlin may reflect the higher affinity of Bmper 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 Bmper (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 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.
of Bmper and Bmp4 and significant involvement of surface Bmp receptors in mediating endocytic sorting of Bmper and Bmp4 to the lysosome to limit the temporal boundaries of Smad activation.

The inhibitory activity of Bmper is supported by our in vivo evidence that Bmper behaves as a tissue-specific Bmp inhibitor in the developing lung of Bmper-deficient mice. The deletion of Bmper affects the distal area of the lung where Bmper 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 Bmper +/- lungs is caused by local deficiency of Bmper and not by a nonspecific secondary effect of delayed lung development. The physiological anti-Bmp activity of Bmper during lung development is completely consistent with our in vitro evidence that Bmper leads to apoptosis of embryonic interstitial lung MEFs, which is normally inhibited by Bmp signaling. Whether Bmper 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 Bmper to Bmp family members likely determine whether Bmper 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. 8d). At low molar concentrations, Bmper 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. 6a). A recent study suggests that this occurs through a Bmp, Bmper, and Bmp receptor tripartite complex that facilitates transfer of Bmp to the Bmp receptor to account for this activity (Serpe et al., 2008). As Bmper 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 Bmper is clearly triggered under these circumstances in a receptor-dependent fashion. The uncleaved form of Bmper 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 Bmper also enhances Bmp4 endocytosis, facilitating a Bmp4 sink through recruitment of Bmps to the cell surface. Furthermore, although the N terminus of Bmper binds Bmp4, the C terminus of Bmper is required for this endocytic mechanism; our results and those of other groups lead us to speculate that the C terminus of Bmper may facilitate endocytosis through interactions with the extracellular matrix and/or Bmp receptors. Under certain conditions, the function of trapping Bmps by Bmper can be uncoupled from an intracellular Bmp sink, and therefore endocytosis is not essential to the inhibitory activity of Bmper. The ability of not only Bmper 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 Bmper, 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. QuikChange site-directed mutagenesis (Agilent Technologies) was used to modify a previously described pSecTag2 vector containing wild-type Bmper (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 using a preembedding immunogold-silver procedure developed by Yi et al. (2001). 70-nm 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 Orius 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, transfected cells were treated with recombinant Bmp4 (0.6 nM) and/or recombinant Bmper, Chordin, Noggin, and/or Gremlin (6.0 nM) in high glucose DME for various times to analyze the temporal effects of Bmper toward Bmp4 activity and internalization. For chase experiments, cells were pulsed with recombinant proteins and then chased with “cold” normal DME and/or Gremlin (6.0 nM) or 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 Bmper or Bmper 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 A0BS; 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-Bmper [R&D Systems], anti-Bmp4 [Milipore], 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 RT (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 tissue
Mouse tissues were fixed overnight in 4% paraformaldehyde/PBS and processed for paraffin embedding. Deparaffinized sections (5 μm) were stained with fluorescein-12-dUTP (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.].

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
Mice deficient for Bmper were generated with standard gene targeting methods (Dai et al., 2003). The targeting construct was generated using pOSf9 (Coran 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 2260 bp cDNA encoding EGF (Clontech Laboratory Inc.); (c) a 300 bp bovine growth hormone poly(A) addition region; 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 tar-g2.0-kb PCR-generated genomic fragment from second intron of the promoter; (b) a 700-bp cDNA encoding EGFP (Clontech Laboratory Inc.); (c) a 4.4-kb PCR-generated fragment from genomic DNA that includes the Mice deficient for Bmper were generated with standard gene targeting approach. 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].

Genotyping of Bmpra mutant mice
Genomic DNA of Bmpra-deficient mice was isolated and digested with HpaI (5’ probe) and Nhel (3’ probe) to identify wild-type and targeted alleles. Bmpra homologous mutants were generated by timed heterozygous matings. A PCR analysis has been developed to genotype the embryos and pups. PCR samples were denatured in 95°C for 60 s, and then subjected to 35 cycles of three-step amplification, a 30-s 94 °C denaturation, 30-s 68 °C annealing, and 45-s 72 °C extension step. A 468-bp product (primers bermf and bergr) represents the wild-type allele and a 455-bp product (primers berkof and bergtr) indicates the target allele. PCR primers: bermf, 5’-TGCGTATCCACCC-CTTGAAATTTCTAG-3’; bergr, 5’-gctctgatgtcttggatc-3’; berkof, 5’-CCAGCCCAACGCTCCCTGCTGAAATCC-3’. 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 RT (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).

Southern blotting analysis
Southern blotting analysis was performed as previously described (Matzuk et al., 1992). Genomic DNA was digested with either HpaI or Nhel and electrophoresed in a 1% agarose gel. Both 5’ and 3’ probe were labeled with α-32P-deoxyadenosine triphosphate by random oligopriming. Membranes containing DNA have hybridized with labeled probes in 65°C for 1 h. The locations of radioactive probe hybridization on membrane were detected by autoradiography.

Statistical methods
Means ± SEM and a one-way analysis of variance were used for all figures that required statistical measurements. A multiple comparison procedure was used, using the Tukey method to determine statistical significance between groups.

Online supplemental material
Fig. S1 support endocytic studies in Fig. 3 through acid wash and Bmp4 neutralization experiments. Fig. S2 shows the strategy for deleting Bmper in ES cells and in mice. Figure S3 shows the immunohistochemistry for GFP to support the Bmper null phenotype in mice lung from Fig. 7. Online supplemental material is available at http://www.jcb.org/cgi/content/full/jcb.200808064/DC1.

We thank Vicky Madden and the Microscopy Services laboratory of the Department of Pathology and Laboratory Medicine for assistance with electron microscopy studies. We thank Willam C. Sessa for the lung endothelial cells null for caveolin-1. We also thank Andrea Portbury for her critical review of the manuscript.

This work was supported by National Institutes of Health grants HL 61655, HL 02658, and HL 072347 to C. Patterson; a Canadian Institute for Health Research grant 178082 to L. Attisano; and a postdoctoral fellowship from the American Heart Association to R. Kelley.

The authors have no conflicting financial interests.

Submitted: 12 August 2008
Accepted: 23 January 2009

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