Bone morphogenetic proteins (BMPs) are members of the transforming growth factor β (TGF-β) superfamily of ligands, which regulate many mammalian physiologic and pathophysiologic processes. BMPs exert their effects through type I and type II serine/threonine kinase receptors and the Smad intracellular signaling pathway. Recently, the glycosylphosphatidylinositol (GPI)-anchored protein DRAGON was identified as a co-receptor for BMP signaling. Here, we investigate whether a homologue of DRAGON, repulsive guidance molecule (RGMa), is similarly involved in the BMP signaling pathway. We show that RGMa enhances BMP, but not TGF-β, signals in a ligand-dependent manner in cell culture. The soluble extracellular domain of RGMa fused to human Fc (RGMa.Fc) forms a complex with BMP type I receptors and binds directly and selectively to radiolabeled BMP-2 and BMP-4. RGMa mediates BMP signaling through the classical BMP signaling pathway involving Smad1, 5, and 8, and it up-regulates endogenous inhibitor of differentiation (Id1) protein, an important downstream target of BMP signals. Finally, we demonstrate that BMP signaling occurs in neurons that express RGMa in vivo. These data are consistent with a role for RGMa as a BMP co-receptor.

Bone morphogenetic proteins (BMPs) represent a large subfamily of the transforming growth factor β (TGF-β) superfamily of ligands, which also includes TGF-β1, -β2, -β3, activins, inhibins, growth and differentiation factors, nodal, Vg1, and Multerian-inhibiting substance (1). These cytokines play a key role in regulating cell proliferation, differentiation, apoptosis, migration, and patterning during development and in adult tissues (2–4). Like other members of the TGF-β superfamily, BMPs exert their effects through a common signal transduction pathway (1). Signaling is initiated when ligand binds to combinations of two type I and two type II serine/threonine kinase receptors. Thus far, three type I receptors (BMPRIA/ALK3, BMPRIIB/ALK6, and ALK2) and three type II receptors (BMP type II receptor (BMPRII), activin type IIA receptor (ActRIIA), and activin type IIB receptor (ActRIIB)) have been identified for BMP subfamily ligands. Ligand binding activates the receptor complex by inducing phosphorylation of the type I receptor by the type II receptor. Phosphorylated type I receptors then phosphorylate receptor-activated Smad1, 5, and 8 (Smad1/5/8). Phosphorylated Smad1, 5, and 8 (p-Smad1/5/8) then form heteromeric complexes with the common mediator Smad (Co-Smad), Smad4, and the activated Smad complexes move to the nucleus, where they act as transcriptional regulators to modulate gene expression. One important target of BMP signals includes the inhibitor of differentiation (Id) gene family, which serve as regulators of growth and differentiation in a variety of tissues (5–9).

Regulation of the signal transduction pathway occurs at many levels. One key regulatory mechanism for many TGF-β superfamily members is through accessory or co-receptors to promote or inhibit ligand binding (1, 10–14). For example, the TGF-β type III receptor (betaglycan) mediates binding of TGF-β2 to the type II receptor and is important for TGF-β2 signaling (10). Glycosylphosphatidylinositol (GPI)-linked proteins from the epidermal growth factor-Cripto-Criptic-FRL-1 family are co-receptors necessary for nodal, Vg1, and growth and differentiation factor 1 signaling (11, 12). Cripto also inhibits activin signaling by preventing binding of the activin type II receptor complex to type I receptors (13). We have recently identified the GPI-anchored protein DRAGON (RGMb) as the first co-receptor for BMP signaling (14). DRAGON enhances cellular responses to BMP, but not TGF-β, signals in a ligand-dependent manner. DRAGON associates with BMP type I and type II receptors, and soluble DRAGON.Fc fusion protein binds selectively to BMP-2 and BMP-4, but not BMP-7 or other members of the TGF-β superfamily of ligands (14). DRAGON is a member of the repulsive guidance molecule (RGM) family of genes, which also includes RGMa and hemojuvelin (HJV/RGMC/HFE2) (15—20). These family members share 50–60% amino acid identity and similar structural features, including an N-terminal signal sequence, conserved proteolytic cleavage site, partial von Willebrand factor type D domain, and GPI anchor (15—20). Unlike DRAGON, RGMa and RGMb are bone-specific proteins.
hemojuvelin also possess an RGD motif, which could be involved in cell attachment (15). RGMa and DRAGON are expressed in a complementary manner in the central nervous system (17–20), where RGMa mediates repulsive axonal guidance (15, 21, 22) and neural tube closure (18), while DRAGON contributes to neuronal cell adhesion through homophilic interactions (17). RGMa also binds to the receptor neogenin (21) and functions as a cell survival factor (23). Hemojuvelin is expressed most heavily in the liver, heart, and skeletal muscle, and is mutated in juvenile hemochromatosis, a disorder of iron overload (16–20, 24).

Here, we investigate whether RGMa is involved in the BMP signaling pathway. Using a reporter assay, we show that transfection of RGMa cDNA into cells enhances BMP, but not TGF-β, signals in a ligand-dependent fashion. Binding and cross-linking studies in a cell-free system demonstrate that soluble RGMa.Fc fusion protein interacts with the BMP type I receptor ALK6 and binds directly to 125I-BMP-2 and 125I-BMP-4, but not other members of the TGF-β superfamily. Co-transfection of RGMa cDNA with dominant negative BMP type I receptors or with dominant negative Smad1 inhibits RGMa-mediated BMP signaling, suggesting that RGMa generates BMP signals via the classical BMP pathway. Transfection of RGMa cDNA into cells induces phosphorylation of endogenous Smad1/5/8 and up-regulates endogenous Id1. Finally, immunofluorescence microscopy of adult rat spinal cord sections, reveals that RGMa is expressed in vivo in neurons, which also show nuclear accumulation of p-Smad1/5/8. Taken together, these data suggest that RGMa functions as a BMP co-receptor.

EXPERIMENTAL PROCEDURES
cDNA Subcloning—cDNA encoding murine RGMa was subcloned into the expression vector pCDNA4/HisB (Promega). cDNA encoding the extracellular domain of murine RGMa was amplified by polymerase chain reaction (PCR) and subcloned into the mammalian expression vector pGigplus (R & D Systems, Minneapolis, MN) into the restriction sites BamHI and HindIII in-frame with the Fc portion of human immunoglobulin (IgG) to generate soluble RGMa.Fc fusion protein.

Cell Culture and Transfection—HEK 293 cells and LLC-PK1 cells were obtained from the American Type Culture Collection (ATCC CRL-1573 and CL-101, respectively) and cultured in Dulbecco's modification of Eagle's medium (DMEM; Cellgro Mediatech, Herndon, VA) containing 10% fetal bovine serum (FBS, Atlanta Biologicals, Lawrenceville, GA). All plasmid transfections were performed with Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. Stably transfected LLC-PK1 cells were selected and cultured in 2 mg/ml G418 (Cellgro Mediatech).

Luciferase Assay—LLC-PK1 cells were transiently transfected with a TGF-β responsive firefly luciferase reporter, (CAGA)₅MLP-Luc (CAGA-Luc, Ref. 25), or a BMP responsive firefly luciferase reporter (BRE-Luc, Ref. 6) (both kindly provided by Peter ten Dijke, Netherlands Cancer Institute), in combination with pRL-TK Renilla luciferase vector (Promega) in a ratio of 10:1 to control for transfection efficiency, with or without co-transfection with RGMa cDNA. Forty-eight hours after transfection, cells were serum-starved in DMEM supplemented with 1% FBS for 6 h and treated with varying amounts of TGF-β1, BMP-2, BMP-4, or BMP-7 ligands (R & D Systems) for 16 h, in the absence or presence of noggin (R & D Systems) or anti-BMP-2/4 antibody (R & D Systems). Cells were lysed, and luciferase activity was determined with the Dual Reporters Assay (Promega) according to the manufacturer's instructions. Experiments were performed in duplicate or triplicate wells. Relative luciferase units (RLU) were calculated as the ratio of firefly (reporter) and Renilla (transfection control) luciferase values.

Reverse Transcription Polymerase Chain Reaction (RT-PCR)—LLC-PK1 cells were grown to confluence on 6-cm tissue culture plates. Total RNA was isolated using the RNeasy Mini Kit (Qiagen, Valencia, CA) including DNase digestion with the RNase-Free DNase set (Qiagen) according to the manufacturer's instructions. First strand cDNA synthesis was performed using iScript cDNA Synthesis kit (Bio-Rad) according to the manufacturer's instructions. Transcripts of BMP-2 were amplified using the forward primer 5'-GTGAGCAAGAGCTTTCGACA-G-3' and reverse primer 5'-GGCATGATTAGTGATGGTCAG-3'. Transcripts of BMP-4 were amplified using the forward primer 5'-AG-
were sonicated and lysed in 200 mM Tris-HCl, pH 8.0, 100 mM NaCl, 1 mM EDTA, 0.5% Nonidet P-40, and 10% glycerol containing a mixture of protease inhibitors (Roche Diagnostics) for twenty minutes on ice. After centrifugation for 20 min at 4 °C, the supernatant was assayed for protein concentration by colorimetric assay (BCA kit, Pierce). 30 µg of protein was separated by SDS-PAGE and transferred to polyvinylidene difluoride membranes. Membranes were probed with α-RGMA (1:2000) as described above for RGMAFc. Membranes were stripped in 0.2 mM glycine, pH 2.5, 0.5% Tween 20 for 1 h, and re-probed in succession with rabbit polyclonal anti-p-Smad1/5/8 antibody (α-p-Smad1/5/8, Cell Signaling, Beverly, MA) (1:1000) at 4 °C overnight according to the manufacturer’s instructions, rabbit polyclonal anti-Smad1 antibody (α-Smad1, Upstate Biotechnology, Lake Placid, NY) (1:250) at 4 °C overnight, mouse monoclonal anti-β-actin antibody (α-β-actin, clone AC 15, Sigma) (1:5000) at room temperature for 1 h, and rabbit polyclonal anti-IId1 antibody (α-IId1, C 20, Santa Cruz Biotechnology) (1:2000) at 4 °C overnight followed by the appropriate horseradish peroxidase-conjugated secondary antibody and chemiluminescence detection after each as described above. Chemiluminescence was quantitated using IPLab Spectrum software (Scanalytics, Vienna, VA).

Northern Blot—Adult rat total RNA was separated on a 1.5% formaldehyde agarose gel and blotted onto GeneScreen Plus membrane (PerkinElmer Life Sciences) as previously described (28).

Immunohistochemistry—Freshly dissected adult rat lumbar spinal cord was embedded in OCT (Sakura, Tokyo, Japan), frozen on dry ice, cut by cryostat in 16-µm sections, and stored at −80 °C. Spinal sections were fixed in 4% paraformaldehyde, washed three times in PBS, and incubated for 1 h at room temperature in blocking buffer (1% bovine serum albumin, 0.5% Triton X in PBS). Fixed sections were incubated overnight at 4 °C in blocking buffer with rabbit polyclonal α-RGMA (1:500) or rabbit polyclonal α-p-Smad1/5/8 (1:100), in combination with mouse monoclonal antineuron-specific nuclear protein antibody (α-NeuN, 1:1000) (Chemicon, Temecula, CA) to visualize neuronal cell bodies. Sections were then washed three times in PBS and incubated with cyanin 3 (Cy3)-conjugated anti-rabbit and fluorescein isothiocyanate (FITC)-conjugated anti-mouse secondary antibodies (1:200 each, Jackson ImmunoResearch) for 1 h at room temperature in blocking buffer (1% bovine serum albumin, 0.5% Triton X in PBS) (16 h in the absence (A and B, bars 1 and 3) or presence of 50 ng/ml BMP-2 (A, bar 2) or 20 ng/ml TGF-β1 (B, bar 2) followed by measurement of luciferase activity. C, LLC-PK1 cells were transfected with BRE-Luc and pRL-TK either alone or with increasing amounts of RGMA cDNA as indicated. Transfected cells were then incubated for 16 h in the absence (white bars) or presence of 50 ng/ml BMP-2 (black bars), followed by measurement of luciferase activity. Luciferase values were normalized for transfection efficiency relative to Renilla activity to generate RLU. Results are reported as the mean ± S.D.

RESULTS

RGMA Mediates BMP, but Not TGF-β, Signaling—Because RGMA family member DRAGON functions as a BMP co-receptor in LLC-PK1 porcine kidney epithelial cells (14), we tested whether RGMA also mediated BMP signaling in these cells. LLC-PK1 cells were transfected with a BMP-responsive firefly luciferase reporter (BRE-Luc, A) or the TGF-β-responsive firefly luciferase reporter (CAGA-Luc, B), in combination with pRL-TK Renilla luciferase vector, either alone (A and B, bars 1 and 2) or with 0.2 µg of RGMA cDNA (A and B, bar 3). Transfected cells were then incubated for 18 h in the absence (A and B, bars 1 and 3) or presence of 50 ng/ml BMP-2 (A, bar 2) or 20 ng/ml TGF-β1 (B, bar 2) followed by measurement of luciferase activity. C, LLC-PK1 cells were transfected with BRE-Luc and pRL-TK either alone or with increasing amounts of RGMA cDNA as indicated. Transfected cells were then incubated for 18 h in the absence (white bars) or presence of 50 ng/ml BMP-2 (black bars), followed by measurement of luciferase activity. Luciferase values were normalized for transfection efficiency relative to Renilla activity to generate RLU. Results are reported as the mean ± S.D.

Production and Characterization of Soluble RGMA.Fc Fusion Protein—To further characterize the role of RGMA in the BMP signaling pathway, we produced RGMA.Fc fusion protein by fusing the extracellular domain of RGMA to the Fc portion of human IgG. We also generated affinity-purified rabbit polyclonal antibody raised against a C-terminal peptide sequence of RGMA upstream of its GPI anchor (α-RGMA). Purified RGMA.Fc was analyzed by reducing SDS-PAGE followed by Western blot using anti-human Fc antibody (α-Fc) and α-RGMA. Both antibodies recognized two bands of 60 and 75 kDa not seen in mock-transfected cells, confirming the presence of both the RGMA and Fc domains, and validating the RGMA antibody (Fig. 3). These bands were not seen when the RGMA antibody was preincubated with competing antigenic peptide (data not shown). The larger band is consistent with the predicted size of the full-length RGMA.Fc fusion protein, and the smaller band is consistent with the predicted size of RGMA.Fc fusion protein.
which has been proteolytically cleaved as previously described for both the chick (15) and mouse homologues (18) of RGMα.

RGMα.Fc Binds Selectively to BMP-2 and BMP-4, but Not BMP-7 or TGF-β1—Next, we tested whether RGMα directly interacted with BMP ligands using soluble RGMα.Fc fusion protein in a cell-free binding system. RGMα.Fc was incubated overnight with $^{125}$I-BMP-2 with or without excess cold BMP-2, -4, -7 or TGF-β1, followed by incubation on protein A-coated plates and determination of radioactivity. RGMα.Fc bound to $^{125}$I-BMP-2 (Fig. 4A, compare bar 2 to 1), and this binding was competitively inhibited by excess cold BMP-2 or BMP-4, but not by BMP-7 or TGF-β1. Similar findings were seen with $^{125}$I-BMP-4 (data not shown).

As supporting evidence for the interaction between RGMα.Fc and BMP-2 and -4, chemical cross-linking experiments were performed using DSS in a cell-free system. $^{125}$I-BMP-4 was cross-linked with RGMα.Fc in the presence of DSS (Fig. 4B, bar 5), and this cross-linking was inhibited by excess cold BMP-4 (Fig. 4B, bar 5). As negative controls, no band was seen in the absence of DSS (Fig. 4B, bars 1 and 2) or when buffer alone (Fig. 4B, bar 3) or ALK5.Fc (a TGF-β type I receptor, Fig. 4B, bar 6) was used in place of RGMα.Fc. Similar results were seen for cross-linking with $^{125}$I-BMP-2 (data not shown). Taken together, these data suggest that RGMα.Fc binds directly and selectively to BMP-2 and BMP-4, but not BMP-7 or TGF-β1.

RGMα Mediates BMP signaling via BMP type I receptors—We next investigated whether RGMα acts via the classical BMP signaling pathway through BMP receptors. Dominant negative mutants of BMP type I receptors ALK3 (ALK3 DN) and ALK6 (ALK6 DN), which are deficient in kinase activity and therefore unable to phosphorylate Smad1/5/8, have been previously described (30, 31). We therefore examined the effects of co-transfection with dominant negative ALK3 and ALK6 mutants on RGMα-mediated BMP signaling. The effect of these mutants on exogenous BMP was also examined. Cells with exogenous BMP-2 increased BRE luciferase activity 6–10-fold above baseline (Fig. 5A, compare bars 2 to 1 and 6 to 5). This stimulation by either RGMα or exogenous BMP-2 was blocked by co-transfection with dominant negative ALK3 (Fig. 5A, bars 3 and 7) or dominant negative ALK6 (Fig. 5A, bars 4 and 8).

To determine whether RGMα interacted directly with BMP type I receptors, purified RGMα.Fc, ALK6.Fc, and/or BMP-2 were incubated in solution either individually or in various combinations, in the presence of the cross-linker DSS. Complexes were pulled-down with protein A beads and analyzed by non-reducing SDS-PAGE, followed by Western blot with
RGMa Is a BMP Co-receptor

**Fig. 5.** RGMa mediates BMP signaling through BMP receptors. A, left panel, LLC-PK1 cells were transfected with BRE-Luc and pRL-TK either alone (bar 1) or in combination with 0.2 μg RGMa cDNA (bars 2–4), in the absence (bar 2), or presence of 1 μg of dominant negative type I receptors ALK3 (ALK3 DN, bar 3) or ALK6 (ALK6 DN, bar 4). A, right panel, LLC-PK1 cells were transfected with BRE-Luc and pRL-TK either alone (bars 5 and 6) or in combination with 1 μg of ALK3 DN (bar 7), or ALK6 DN (bar 8), followed by incubation in the absence (bar 5) or presence of 50 ng/ml BMP-2 (bars 6–8). Luciferase activity was measured from cell extracts and normalized for transfection efficiency relative to Renilla activity to generate RLU. Results are expressed as the mean ± S.D. B, 200 ng of RGMa, 200 ng of ALK6-Fc, and/or 100 ng of BMP-2 were incubated in solution in various combinations as indicated with the cross-linker DSS. Complexes were pulled down with protein A beads, and the protein complex was analyzed by non-reducing SDS-PAGE followed by Western blot with RGMa antibody. Arrowheads indicate slower migrating bands containing RGMa-Fc complexes. C, buffer alone (bar 1), 10 ng of RGMa-Fc alone (bar 2), 10 ng of ALK6-Fc alone (bar 3), or the combination of 10 ng each of RGMa-Fc and ALK6-Fc (bar 4) were incubated with [125I]-BMP-2, followed by incubation on protein A-coated plates and determination of radioactivity using a standard γ counter.

α-RGMa. As previously shown, RGMa-Fc formed a complex in solution with BMP-2, demonstrated by a more slowly migrating band under non-reducing conditions compared with RGMa-Fc alone (Fig. 5B, compare arrowhead in lane 4 to lane 3). RGMa-Fc also formed a complex with ALK6-Fc, even in the absence of BMP-2 (Fig. 5B, compare arrowhead in lane 5 to lane 3). In the presence of BMP-2, an even larger shift was seen, suggesting the possible formation of a complex containing RGMa-Fc, ALK6-Fc, and BMP-2 (Fig. 5B, lane 6, arrowhead). No bands were seen for ALK6-Fc or BMP-2 in the absence of RGMa-Fc (Fig. 5B, lanes 1 and 2).

BMP ligands exhibit high affinity for BMP type I receptors and low affinity for BMP type II receptors (1). We therefore tested whether the combination of RGMa and BMP type I receptors increased binding of BMP ligands compared with BMP type I receptors alone. Purified RGMa-Fc and ALK6-Fc were incubated overnight in solution with [125I]-BMP-2, followed by incubation on protein A-coated plates and determination of radioactivity. RGMa-Fc and ALK6-Fc alone each significantly bound [125I]-BMP-2 (Fig. 5C, compare bars 2 and 3 to 1). As a negative control, BMP type II receptor was unable to bind (data not shown). The combination of RGMa-Fc and ALK6-Fc increased binding to [125I]-BMP-2 compared with ALK6-Fc alone (Fig. 5C, compare bar 4 to 3).

**Fig. 6.** RGMa mediates BMP signaling through Smad1/5/8 and up-regulates Id1. A, LLC-PK1 cells were transfected with BRE-Luc and pRL-TK either alone (lanes 1, 7–8), or in combination with 1 μg of WT Smad1, bar 2 and 9) or 1 μg of dominant negative Smad1 (Smad1 DN, bars 3 and 10). Transfected cells were then incubated in the absence (bars 1–3 and 7) or presence of 50 ng/ml BMP-2 (bars 8–10). Alternatively, cells were co-transfected with BRE-Luc, pRL-TK, and 0.2 μg of RGMa alone (bar 4), or in combination with Smad1 WT (bar 5) or Smad1 DN (bar 6). Luciferase activity was measured from cell extracts and normalized for transfection efficiency relative to Renilla activity to generate RLU. Results are reported as the mean ± S.D. B, LLC-PK1 cells were transiently transfected with 5 μg of RGMa cDNA (middle 3 lanes) or empty vector (left and right 2 lanes). 24 h after transfection, cells were incubated without (lanes 1–5) or with 50 ng/ml BMP-2 (right two lanes) for 2 h. Cell lysates were analyzed by Western blot in succession with RGMa antibody (α-RGMa), phosphorylated Smad1/5/8 antibody (α-p-Smad1/5/8), Smad1 antibody (α-Smad1), a previously described dominant negative Smad1 (Smad1 DN, bar 7), or BMP type II receptor was unable to bind (data not shown). As a loading control, Id1 antibody (α-Id1), and actin antibody (α-β-actin, as a loading control). C and D, chemiluminescence from the Western blot in B was quantitated by IPLab Spectrum software for phosphorylated Smad1/5/8 relative to Smad1 expression (C) and Id1 relative to β-actin expression (D). Results are reported as the mean ± S.D. of control cells (C), cells transfected with RGMa, and cells treated with BMP-2.

RGMa Mediates BMP Signaling via Smad1/5/8, and Up-regulates Endogenous Id1 Expression. To further explore whether RGMa acts via the classical BMP signaling pathway, we studied the effects of wild-type Smad1 (WT Smad1) versus dominant negative Smad1 (DN Smad1), a previously described dominant negative mutant of Smad1 with deleted C-terminal phosphoacceptor residues (14, 32, 33), on RGMa-mediated BMP signaling. Results were compared with their effects on exogenous BMP-2 signaling as a control. Consistent with prior studies (14, 34, 35), transfection with WT Smad1 alone increased BRE luciferase activity 8-fold above baseline (Fig. 6A, compare bar 2 to 1), while transfection with DN Smad1 alone decreased BRE luciferase activity below baseline (Fig. 6A, compare bar 3 to 1). Transfection with RGMa increased BRE luciferase activity 7-fold above baseline (Fig. 6A, bar 4). Co-transfection of WT Smad1 with RGMa further augmented the signaling induced by either WT Smad1 or RGMa alone (Fig. 6A, compare bar 5 to 2, 4).
RGMa blocked the increase in signal seen with RGMa alone (Fig. 6A, compare bar 6 to 4). Similar results were seen for the effects of WT Smad1 and DN Smad1 on exogenous BMP-2 stimulation (Fig. 6A, bars 7–10).

As supporting evidence that RGMa mediates BMP signaling through the Smad signaling pathway, we examined the effect of RGMa expression on phosphorylation of endogenous Smad1/5/8. LLC-PK1 cells were transiently transfected with RGMa cDNA and cell lysates were assayed for p-Smad1/5/8 by Western blot (Fig. 6B, α-p-Smad1/5/8). Blots were stripped and reprobed for total Smad1 as a loading control (Fig. 6B, α-Smad1). Results were compared with mock-transfected cells as a negative control, and cells stimulated for 2 h with 50 ng/ml exogenous BMP-2 as a positive control. Expression of p-Smad1/5/8 relative to Smad1 was quantitated using IPLab Spectrum software (Fig. 6C). Consistent with our prior data supporting the notion of endogenous BMP signaling in these cells, mock-transfected cells (without RGMa or exogenous BMP-2 stimulation) did have some basal level of p-Smad1/5/8 (Fig. 6B, left two lanes). Transfection with RGMa cDNA increased p-Smad1/5/8 levels compared with mock-transfected cells (Fig. 6B, compare middle three lanes to left two lanes; Fig. 6C, compare bar 2 to 1). As a positive control, stimulation with exogenous BMP-2 also increased p-Smad1/5/8 levels (Fig. 6B, right two lanes; Fig. 6C, bar 3).

We then investigated whether RGMa affected expression of endogenous Id1, an important downstream target of BMP signals (5–9). Western blots used in the p-Smad1/5/8 assay above were stripped and re-probed with anti-Id1 antibody (Fig. 6B, α-Id1). Blots were stripped again and re-probed with actin antibody (Fig. 6B, α-β-Actin) as a loading control, and the ratio of Id1 to β-actin expression was quantitated using IPLab Spectrum software (Fig. 6D). Transfection with RGMa increased expression of Id1 protein about 2.3-fold compared with mock-transfected cells (Fig. 6B compare middle three lanes to left two lanes; Fig. 6D compare bar 2 to 1). As a positive control, stimulation with exogenous BMP-2 also increased Id1 expression (Fig. 6B, right two lanes; Fig. 6D, bar 3). Thus, RGMa mediates BMP signaling via the classical BMP pathway involving Smad1/5/8, and RGMa increases expression of endogenous Id1 protein, a downstream target of BMP signals.

**RGMa Is Widely Expressed**—Previous studies have focused on detailing the expression pattern of endogenous RGMa in the central nervous system and during development (17–20). To begin to elucidate the role of RGMa in vivo, we performed Northern blot analysis of endogenous RGMa expression in a variety of adult rat tissues. RGMa message is widely expressed in many of the tissues tested, including heart, brain, lung, liver, skin, kidney, and testis (Fig. 7). Two distinct bands were seen in some tissues, possibly representing alternative transcription initiation or alternative splicing.

**BMP Signaling Occurs in Neurons of the Adult Spinal Cord Which Express RGMa**—Next, we explored whether RGMa expression in vivo correlates with its hypothesized role as a co-receptor for BMP signaling. RGMa mRNA has been previously documented to be widely expressed in a complementary fashion to DRAGON in the central nervous system (17–20), including ventral horn neurons of the spinal cord (17). We therefore determined whether RGMa protein was expressed in ventral horn neurons, and we examined whether these neurons also showed evidence of BMP signaling, i.e., nuclear accumulation of p-Smad1/5/8. Adult rat spinal cord sections were analyzed by immunofluorescence microscopy with α-RGMa, α-p-Smad1/5/8, and/or anti-neuron-specific nuclear protein antibody (α-NeuN) to visualize neuronal cell bodies (17). RGMa staining colocalized with NeuN staining in ventral horn motor neurons (Fig. 8, panels A–C). Ventral horn motor neurons were also positive for nuclear p-Smad1/5/8 (Fig. 8, panels D–F), suggesting that there is basal signal transduction via the BMP pathway in these cells. Thus, endogenous RGMa is expressed in ventral horn motor neurons, which also generate BMP signals, consistent with a role for RGMa as a BMP co-receptor in vivo.

**DISCUSSION**

BMPs are members of the TGF-β superfamily of ligands, which play a pleitropic role in vertebrate development and adult tissues (2–4). These functions require tight spatiotemporal regulation and specific activation via receptor complexes of particular intracellular signaling pathways. In order to generate specificity and to finely tune these signals, regulation occurs at multiple levels extracellularly, at the membrane surface, and intracellularly (1, 4, 36).

For the BMP pathway, most regulatory mechanisms identified to date are inhibitory. Soluble BMP antagonists such as noggin, chordin, chordin-like, follistatin, FSRP, DAN, cerebroid, and gremlin bind BMPs in the extracellular space and mask receptor binding interfaces for BMP type I and type II receptors (4, 29). At the membrane surface, Bambi (BMP and activin receptor membrane-bound inhibitor), which is structurally related to type I receptors in the extracellular domain but lacks the intracellular serine/threonine kinase domain, inhibits BMP signals by stably associating with type II receptors and pre-
ventiling formation of the active receptor complexes (37). Inhibin, in concert with its co-receptor betaglycan (also known as the TGF-β type III receptor), competes with BMPs for access to BMP type II receptors (38). Inside the cell, inhibitory Smads (Smad 6 and Smad 7) inhibit signaling by either interacting with phosphorylated type I receptors to prevent activation of receptor-activated Smads (39–41), or through competition to prevent formation of the receptor-activated Smad/Co-Smad complex (42). Other intracellular molecules Smurf1 and Smurf2 (Smad ubiquitination regulatory factors), selectively target-activated type I receptors and Smad proteins for degradation (43–45).

For other TGF-β superfamily members, accessory or co-receptors also play an important regulatory role to promote or inhibit ligand binding (1, 10–13). Recently, we identified DRAGON (RGMb) as the first known co-receptor for BMP signaling (14). We therefore investigated whether another RGM family member, RGMa, was similarly involved in the BMP signaling pathway. Here, we have demonstrated that RGMa is a BMP co-receptor, which enhances cellular responses to BMP, but not TGF-β.

Although transfection of RGMa into LLC-PK1 cells enhanced BMP signal transduction without exogenously added ligand, our results suggest that this is a ligand-dependent process. Ligand dependence is supported by the fact that RGMa-mediated BMP signaling was inhibited by noggin, a soluble BMP inhibitor which binds and sequesters BMP ligands, preventing access to BMP receptors (4, 29). However, this does not pinpoint the endogenous ligand(s) responsible for RGMa-mediated BMP signaling in these cells, because noggin has been shown to bind and antagonize several BMPs, including BMP-2, BMP-4, and BMP-7, as well as some other TGF-β superfamily members, including growth and differentiation factor 5 (4, 29, 46, 47). Our findings that a neutralizing antibody to BMP-2 and BMP-4 also inhibited RGMa-mediated BMP signaling suggest that the major endogenous ligand(s) in these cells may be BMP-2 and/or BMP-4. Indeed, RT-PCR confirmed that these cells do endogenously express both BMP-2 and BMP-4. However, this does not preclude the possibility that some portion of the signaling is related to other BMP ligands. Indeed, the manufacturer of this neutralizing antibody does report some minimal cross-reactivity to other BMP ligands. Additionally, this antibody did not completely inhibit RGMa-mediated BMP signaling to baseline levels.

Further evidence for the role of RGMa as a co-receptor for BMP-2 and/or BMP-4 is provided by binding and cross-linking studies of purified RGMa.Fc in solution. These assays allow the determination of the binding properties of single types of receptors and combinations of receptors in isolation, avoiding the presence of any confounding co-expressed accessory proteins that may also be present at the cell surface (26). In these studies, RGMa.Fc bound directly and specifically to 125I-BMP-2 and 125I-BMP-4, and this binding was competitively inhibited by excess cold BMP-2 and BMP-4, but not BMP-7 or TGF-β1. RGMa.Fc also formed a complex with the BMP type I receptor ALK6.Fc, and the presence of RGMa.Fc in combination with ALK6.Fc increased binding to 125I-BMP-2 compared with ALK6.Fc alone. No further increase in binding was seen with the addition of the BMP type II receptor.Fc (data not shown). Although this increased binding was additive, not synergistic, the fact that RGMa.Fc formed a complex with ALK6.Fc raises the possibility that on the cell surface, RGMa may associate with BMP type I receptors, thereby increasing overall binding of BMP ligands to the receptor complex and enhancing BMP signal transduction. However, the detailed mechanism by which RGMa enhances BMP signaling remains to be fully elucidated.

While the binding and cross-linking experiments were performed using RGMa.Fc and BMP receptor.Fc fusion proteins in solution, support for the role of GPI-anchored, cell-surface RGMa in the BMP signaling pathway is provided by our findings that RGMa-mediated BMP signaling was inhibited by dominant negative BMP type I receptors and by dominant negative Smad1, using a BMP-responsive luciferase reporter assay in cell culture. Additionally, transfection of RGMa into LLC-PK1 cells increased phosphorylation of endogenous Smad1/5/8, and increased expression of endogenous Id1 protein, an important target gene of BMP signaling in many tissues (5–9). The physiologic role of endogenous RGMa as a BMP co-receptor in vivo is supported by our finding that RGMa was expressed in spinal cord neurons, which also showed nuclear accumulation of p-Smad1/5/8, indicative of BMP signal transduction in these cells. Future studies will be needed to determine the functional significance of BMP signaling in these cells. Future studies will also be needed to determine whether the role of RGMa as a BMP co-receptor is independent of, or related to, its other known functions in mediating axonal repulsive guidance (15, 22) and neural tube closure (18). Interestingly, RGMa also acts as a cell survival factor by binding to the receptor neogenin and inhibiting the neogenin pro-apoptotic activity (21, 23). Whether RGMa binding to neogenin is affected by BMPs, or conversely, whether RGMa binding to BMPs is affected by neogenin, remains unknown.

RGMa and DRAGON are members of the RGM family of proteins, which also includes the juvenile hemochromatosis gene HJV. RGM family members are highly conserved across vertebrates and invertebrates and share significant sequence homology as well as similar structural features (15–20). Preliminary data suggest that HJV also mediates BMP signaling,3 indicating that these family members all share the ability to act as co-receptors to enhance BMP signals.

Our results do not reveal any differences between RGMa and DRAGON in regard to their function as BMP co-receptors. Both bind to BMP-2 and BMP-4, but not BMP-7 or other members of the TGF-β superfamily. Both signal via the BMP type I receptors ALK3 and ALK6 and Smad1. Thus, it remains unclear what differentiates these family members with regard to their role as BMP co-receptors, and why different family members have evolved. Further studies will be needed to determine the specificity and affinity of RGM family members for interactions with the full range of BMP ligands and receptors. Whether additional co-receptors exist for other BMP ligands remains unknown. Interestingly, a secreted protein, Kielin/chordin-like protein, has recently been described as a paracrine enhancer of BMP-7 signaling (48). Thus, multiple enhancing regulatory mechanisms may exist for BMP signaling to complement the inhibitory regulatory mechanisms that have been described (1, 4, 36).

The role of RGM family members may be to differentially increase the sensitivity of cells in which they are expressed to low levels of BMP ligands, thus contributing to the tight spatiotemporal regulation of BMP signal transduction. Northern blot analysis of adult rat tissues revealed that endogenous RGMa is expressed in a variety of organs, including heart, brain, lung, liver, skin, kidney, and testis. Although prior studies have reported a more limited distribution, they were largely focused on expression in the central nervous system and during development (17–20). Additionally, the one previously published Northern blot of RGMa expression in a variety of tissues was limited by underexposure (18), also a possible explanation for their finding only the larger of the two bands seen in our

3 J. L. Babitt, unpublished data.
study. Recent work has also demonstrated a broader tissue distribution for DRAGON, including many tissues throughout the reproductive axis (49). We have also found DRAGON expression in the adult rat heart, liver, and kidney by Northern blot (data not shown). While HJV expression has been described predominantly in the liver, cardiac muscle, and skeletal muscle (16–20), one recent study suggested that HJV is also expressed in the adult mouse brain, lung, spleen, kidney, testis, blood, stomach, and intestine (24). Thus, RGM family member expression appears to overlap in a variety of tissues. However, it is possible that the distribution within those tissues is different. For example, in the central nervous system, where RGMa and DRAGON expression have been best characterized, they are predominantly expressed in non-overlapping areas (17–20).

Our results help to define the first family of proteins which function as BMP co-receptors. We hypothesize that RGM family members increase the sensitivity of cells in which they are expressed to BMP stimulation, thereby allowing these cells to respond earlier or more robustly to a low level of BMP ligand. RGM family members thus represent an important addition to the complex array of regulatory molecules which help to generate specificity and tightly coordinate cellular responses to BMP ligands.

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REFERENCES
1. Shi, Y., and Massague, J. (2003) Cell 113, 685–700
2. Hogan, B. L. (1996) Trends Genet. 12, 260–266
3. Zhao, G. Q. (2003) J. Biol. Chem. 278, 19842–19849
4. Valdiviezo, L., and Massague, J. (1996) J. Biol. Chem. 271, 3176–3185
5. Miyazono, K., and Miyazawa, K. (2002) Sci. STKE PE40
9. ten Dijke, P., Korchynskyi, O., Valley, et al. (2004) J. Biol. Chem. 279, 1605–1610