Bone morphogenetic proteins (BMPs), a subset of the transforming growth factor (TGF)-β superfamily, regulate a diverse array of cellular functions during development and in the adult. BMP-9 (also known as growth and differentiation factor (GDF)-2) potently induces osteogenesis and chondrogenesis, has been implicated in the differentiation of cholinergic neurons, and may help regulate glucose metabolism. We have determined the structure of BMP-9 to 2.3 Å and examined the differences between our model and existing crystal structures of other BMPs, both in isolation and in complex with their receptors. TGF-β ligands are translated as precursors, with pro-regions that generally dissociate after cleavage from the ligand, but in some cases (including GDF-8 and TGF-β1, -2, and -3), the pro-region remains associated after secretion from the cell and inhibits binding of the ligand to its receptor. Although the pro-region of BMP-9 remains tightly associated after secretion, we find, in several cell-based assays, that the activities of BMP-9 and BMP-9-pro-region complex were equivalent. Activin receptor-like kinase 1 (ALK-1), an orphan receptor in the TGF-β family, was also identified as a potential receptor for BMP-9 based on surface plasmon resonance studies (BIACore) and the ability of soluble ALK-1 to block the activity of BMP-9-pro-region complex in cell-based assays.

Transforming growth factor β (TGF-β)\(^1\) signaling controls a wide variety of processes over the lifetime of an organism. A subset of this large and well conserved family is the group of bone morphogenetic proteins (BMPs) and growth and differentiation factors (GDFs), which regulate a diverse array of cellular functions, including differentiation, proliferation, organogenesis, axon guidance, apoptosis, and the establishment of left-right asymmetry \((1–3)\). BMPs and GDFs are highly conserved throughout the animal kingdom, with examples ranging from Drosophila to humans. They have frequently been implicated in the treatment of bone disorders and injury, in accordance with their robust ability to generate de novo bone formation.

All TGF-β ligands are translated as precursor proteins, consisting of an amino-terminal pro-region and a carboxyl-terminal ligand. This precursor forms a disulfide-linked homodimer in the cytoplasm, and the pro-region is then cleaved from the ligand. In most cases, the pro-region disassociates, and the mature ligand is secreted from the cell, but the pro-regions of GDF-8 (also known as myostatin) and TGF-β1, -2, and -3 remain noncovalently associated with the ligand after secretion and inhibit binding of their ligands to their respective receptors \((4–6)\). Transgenic mice overexpressing the pro-region of GDF-8 show dramatic increases in muscle mass, further indicating that the pro-region functionally inhibits GDF-8 \((7)\). The pro-region of BMP-9 also remains tightly associated after secretion from the cell.

BMP signaling is induced when a dimeric ligand binds to the extracellular domains of two type I and two type II receptors \((8)\). This assembly brings the intracellular domain of both receptor types into close proximity, permitting the constitutively active intracellular kinase domain of the type II receptor to cross-phosphorylate the intracellular Gly-Ser (GS) domain of the type I receptor \((9)\). Receptor-regulated Smad proteins are phosphorylated by the activated type I receptor kinase and associate with Smad4. This complex translocates to the nucleus, interacting with various cofactors to modify gene expression \((10)\).

BMP activity is functionally regulated in a variety of ways, including cell-specific production and timely secretion of BMP ligands \((11)\), proteolytic cleavage of precursors into active ligands \((12)\), sequestering of active ligands by inhibitory molecules, including noggin, chordin, and follistatin \((13)\), inhibition of ligands by various pro-regions \((4–7,14)\), and tissue-specific RNA splicing of receptors \((15–17)\).

In the adult rat, BMP-9 is expressed predominantly in the liver and has been shown to induce proliferation of cultured liver cells \((18)\). BMP-9 mRNA was also found in the septum and spinal cord of E13 mice. In vitro and in vivo, BMP-9 was found to promote cholinergic differentiation and the synthesis of acetylcholine and effectively maintained the cholinergic phenotype of differentiated cells \((19)\). Other studies indicate that BMP-9 produces ectopic bone growth and potently directs the differentiation of mesenchymal cells into cartilage \((20–22)\).
Crystal Structure of BMP-9

More recently, BMP-9 was identified as a regulator of glucose metabolism by modulating the transcription of several genes that are involved in glucose and fatty acid metabolism, decreasing glucose production in cultured cells, and reducing glycemia in diabetic mice (23). Activin-like kinase-1 (ALK-1) is an orphan receptor in the TGF-β family. It has been implicated as an inhibitor of lateral TGF-β/ALK-5 signaling (24), correlated with vasculosclerosis and angiogenesis (25), and may be a factor in hereditary hemorrhagic telangiectasia (26).

The stereotypical scaffold of BMPs consists of a cysteine knot characterized by three pairs of highly conserved disulfide bonds. This fold has been previously described as a “hand” with a concave “palm” side and two parallel β-sheets forming the “fingers.” In the mature, dimeric ligand, these two β-sheet fingers extend from the cysteine core of the protein like butterfly wings. Binding to type I receptors occurs near the α-helix on the concave side at the junction between the two subunits (27), whereas binding to the type II receptors is located on the convex side of the hand near the “fingertips” (28, 29).

We report here the structure of BMP-9 at 2.3 Å and examine structural differences between BMP-9 and BMP-2, BMP-7, the BMP-2-BMPR-IA complex, and the BMP-7-ACTR-IIB complex. Results of in vitro cell-based assays indicate that both BMP-9 and BMP-9-pro-region complex are equally active in signaling and cell-growth stimulation, whereas the pro-region alone is inactive. Based on surface plasmon resonance and functional inhibition in several cell-based assays, we suggest activin receptor-like kinase-1 (ALK-1) as a functional receptor for BMP-9.

EXPERIMENTAL PROCEDURES

Materials—All recombinant receptor:Fc chimeras were purchased from R & D Systems and were reconstituted as instructed by the manufacturer. BLAcore instrumentation, software, and CM5 sensor chips were purchased from BIACORE™ (Uppsala, Sweden). Fetal bovine serum and all other cell culture media were purchased from Invitrogen unless otherwise stated.

Cell Lines—Rat hepatoma cell line H4IIE, mouse myoblast cell line C2C12, and mouse pre-adipocyte cell line 3T3-L1 were all obtained from the American Type Culture Collection (ATCC, Manassas, VA). These cell lines were propagated in media recommended by ATCC. The dihydrololate reductase-deficient Chinese hamster ovary cell line DG44 (30) was propagated in minimal essential α medium, 5% dialyzed fetal bovine serum, and 4 mM l-glutamine. Selections were performed in minimal essential α medium lacking ribonucleotide and deoxyribonucleotide. Cells were supplemented with 5% dialyzed fetal bovine serum and 4 mM glutamine. Methotrexate (Sigma) was used at the concentrations indicated.

Protein Expression and Purification—The coding sequence for full-length BMP-9 with pro-region was cloned into pc4, a proprietary mammalian expression vector, and the construct was transfected into dihydrololate reductase-deficient Chinese hamster ovary cell line DG44 using Lipofectamine 2000 (Invitrogen) according to the manufacturer’s protocol. Typically, ~1 × 10^6 cells were transfected with 5 μl of Lipofectamine 2000, 5 μg of pc4.BMP-9, and 0.5 μg of psV2.NEO plasmids. The transfected cells were put into selection after ~36 h in minimal essential α medium containing 25 mM methotrexate and 1 mg/ml geneticin. After ~2–3 weeks, single colonies were isolated and seeded into 24-well plates. When confluent, the supernatants from these clones were screened for BMP-9 activity using cell-based reporter assays. The high expressing clones were selected and amplified stepwise up to 0.5 mM methotrexate. For large scale production of BMP-9, a high expressing clone was grown in Chinese hamster ovary-5 medium (a Human Genome Sciences proprietary serum-free medium without insulin) for 5 days, and the conditioned medium was harvested.

To purify the recombinant dimer of BMP-9 culture supernatant was adjusted to pH 6.4 with pH 5.0, 1 mM MES, and diluted to a conductivity of <7–8 μS. The sample was loaded on a 50-mL POROS 50 HS (Perseptive Biosystems) column that had been pre-equilibrated with pH 6.4, 20 mM MES, and 50 mM NaCl. BMP-9 complex was eluted with a 20-column-volume pH gradient from pH 6.4 to 8.0, with pH 8.0 buffer containing 20 mM Tris-HCl and 50 mM NaCl. The fractions of partially purified BMP-9-pro-region complex were pooled and adjusted to pH 7.4 with pH 9.0, 1 mM Tris, and diluted to the conductivity <5 μS. The sample was loaded onto an 8-mL MonoQ column (Amersham Biosciences) equilibrated with pH 7.4 and 20 mM Tris. BMP-9-pro-region complex eluted was eluted by a 15-column-volume salt gradient of 0–0.5 mM NaCl.

For cell-based assays, BMP-9 dimer was separated from its pro-region using 50% cold ethanol precipitation. After cold precipitation, the pro-region and remaining BMP-9-pro-region complex were spun down, leaving isolated, dimeric BMP-9 in the supernatant. The precipitation was repeated once after dissolving the pro-region and BMP-9-pro-region complex in pH 7.4, 20 mM Tris, 200 mM NaCl. The pro-region was further separated from BMP-9-pro-region complex on a MonoQ column using the same procedure as used to purify the complex. Soluble BMP-9 in the supernatant was concentrated using a Centricon (Amicon), and the buffer was changed to pH 7.4, 20 mM Tris, 200 mM NaCl, 30% ethanol.

Proprietary and SDS gels, BMP-9 dimer and pro-region were isolated by reverse phase chromatography, using a C4 high pressure liquid chromatography column (Vydac) and eluted with a 27–42% acetonitrile gradient. BMP-9 dimer eluted at 31% acetonitrile, and the pro-region eluted at 35% acetonitrile. Fractions were frozen in liquid nitrogen, lyophilized, and reconstituted in water.

Crystallization, Data Collection, and Refinement—BMP-9 was crystallized from a solution containing BMP-9 dimer, BMP-9 monomer, pro-region, and other secreted proteins in a condition identified from sparse matrix (Hampton) screening. Total protein concentrations ranged from 3.8 to 9 mg/ml, with BMP-9 dimer constituting ~15–20% of the total protein. Crystals of BMP-9 were obtained by hanging drop at 23 °C with a well solution of 1–1.2 M NaCl, 7–10 mM hexadecyltrimethylammonium bromide, and 10 mM MgCl2. Proprietary crystals of BMP-9 were soaked in mother liquor supplemented with 12–15% glycerol as a cryoprotectant. Crystals were flash frozen in liquid nitrogen, and diffraction data were obtained at Stanford Synchrotron Radiation Laboratory on beam line 9.2. All integration and scaling were performed with HKL2000. The space group was found to be 14/22, with unit cell dimensions a = b = 71.23, 144.90, α = β = γ = 90°. A molecular replacement solution was found from the structure of BMP-2 monomer as the search model. The asymmetric unit contains one copy of the BMP-9 monomer, leaving a solvent content of 67.6%. The protein was manually rebuilt in O (32), and the structure was refined using Refmac5 (Collaborative Computational Project 4) and crystallography NMR software (33). Four translation, libration, and screw rotation groups were used, dividing the protein into regions from Ala6 to Ala15, Tyr15 to His32, Ala33 to Met164, and Leu164 to Gly315.

Size Exclusion Chromatography—Approximately 100 μg of total protein from Chinese hamster ovary cell media was run in 10 mM Tris, 100 mM NaCl over an S-200 size exclusion chromatography column.

Native Gels and SDS-PAGE—Native gels were 6% polyacrylamide, pH 8.8, and run at 27 mA. Proteins were mixed to final concentrations of 0.1–1 mg/ml and allowed to equilibrate for at least 15 min at room temperature or overnight at 4 °C before loading. Non-reducing SDS gels were 12% polyacrylamide and run at 200 mV.

Alkaline Phosphatase Assay—Mouse pluripotent C2C12 cells were cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum, 4 mM l-glutamine, 1.5 g/liter sodium bicarbonate, 4.5 g/liter glucose, 100 IU/ml penicillin, and 100 μg/ml streptomycin. For the alkaline phosphatase assay, C2C12 cells were seeded in 96-well tissue culture plates at 1,000 cells/well in 100 μl of medium. The following day, the medium was removed and replaced with treatments in Dulbecco’s modified Eagle’s medium containing 0.1% fetal bovine serum. The cells were cultured for 4 days and conditioned media were collected for measurement of alkaline phosphatase activity. In inhibitor studies, soluble receptors were added at the indicated concentrations with BMP-9-pro-region complex (5 μM) or BMP-4 (4.7 μM). Alkaline phosphatase activity was measured using the Phospho-Light™ System (Applied Biosystems) according to the manufacturer’s directions. Briefly, the cells were rinsed with phosphate-buffered saline, lyzed in buffer containing 0.2% Triton X-100, incubated 5 min in assay buffer and 20 min in reaction buffer containing CSPD (disodium 3-(4-methoxyphenyl)phenylphosphinic acid), 10 mM MgCl2, and 0.5 mM molybdate (34). The luminescent signal was read using a luminometer (Applied Biosystems). All treatments were performed in triplicate. The average and S.D. were determined and data plotted using Prism software (GraphPad Software, Inc., San Diego, CA).
**RESULTS**

BMP-9 was crystallized and solved with molecular replacement at a resolution of 2.32 Å (Table I). BMP-9 shows the characteristic cysteine knot scaffold and overall butterfly-like conformation, with an α-helix ("knuckle," a3) epitope and two β-stranded sheets (fingers F1 and F2) extending from the core of the molecule (Fig. 1A), but it also deviates from known BMP structures in regions known to be important for receptor binding.

Previous structural studies of BMP-2 in complex with BMPR-IA ectodomain revealed the residues likely to be in-
volved in determining binding affinity and specificity (defined by a 4.0-Å cutoff and colored green in Fig. 1). To examine which structural differences between BMP-2 and BMP-9 might influence receptor binding, BMP-2/BMPR-IA (Protein Data Bank (PDB) entry 1ES7) was aligned with BMP-9 using least squares minimization. This comparison reveals the regions of the ligands that are likely to be significantly different, particularly the pre-helix loop.

To quantify differences between BMP-9 and BMP-2, C-alpha r.m.s. deviations between BMP-9 and free (uncomplexed) BMP-2 (PDB entry 3BMP) were calculated. The average r.m.s. deviation for the monomer is 1.50 Å. Of the amino acids likely to be involved in binding a type I receptor, C-alpha r.m.s. deviations 1 S.D. above the mean occur between residues Gly27 in BMP-2 and Gly21 in BMP-9 and the segments Phe49–Asp53 in BMP-2 and Phe43–Asp47 in BMP-9. One residue pair (Pro50 in BMP-2 and Pro44 in BMP-9) at the type I interface has an r.m.s. deviation 1.5 S.D. from the mean. All of these residues are located in the pre-helix loop, or “wrist” epitope (colored red in Fig. 1A).

The crystal structure of BMP-7 in complex with activin receptor IIA (ActR-IIA, PDB entry 1LX5) has previously been solved, and the residues likely to be involved in binding (defined by a 4.0-Å cutoff) are colored red in Fig. 1C. Root mean square deviations were also calculated between BMP-9 and free BMP-7 (PDB entry 1LXI). One position at the type II binding interface, Ala58 in BMP-7 and Ala28 in BMP-9, was found to have the most pronounced difference from the mean r.m.s. deviation (1.76 Å).

In a sequence alignment of BMP-9 and BMP-2 (Fig. 2), sequence differences in binding regions identified by structural and mutational (27) studies are most notable in residues Asn59 in BMP-2 and Lys53 in BMP-9 (acidic to basic) and in residues His64 in BMP-2 and Asp68 in BMP-9 (basic to acidic). A comparison between the amino acid sequences of BMP-9 and BMP-7 (Fig. 2) also reveals significant changes, including Tyr44 in BMP-7 to Arg4 in BMP-9 (aromatic to basic), Glu60 in BMP-7 to Lys30 in BMP-9 (acidic to basic), and Gly61 in BMP-7 to Glu31 in BMP-9 (aliphatic to acidic) at the type II binding interface. Based on the BMP-7/ActR-IIB structure (28), Tyr44 and Gly61 both interact with the acidic Asn65, and Glu60 interacts with basic Lys76. These data indicate that differences between BMP-7 and BMP-9 in binding affinity for type II receptors are most likely because of amino acid changes at the binding interface, although type II receptors are generally quite promiscuous (37).

BMP-9, when purified from cell medium, remains noncovalently associated with its pro-region. This complex runs as a single peak by size exclusion chromatography, with a retention volume consistent with a complex of one BMP-9 dimer and two pro-regions (Fig. 3A). An SDS gel shows clean separation after reverse-phase chromatography (Fig. 3B). When separated, BMP-9 and pro-region are recombined in equimolar quantities and run on a native gel, a band similar to what is seen in the original protein solution appears, and neither the isolated dimeric BMP-9 band nor the isolated pro-region band is observed, indicating that the complex can be reformed after separation (Fig. 3C).
but had no effect on BMP-9-pro-region complex. Of the other soluble receptor:Fc chimeras tested, only BMPR-II:Fc partially blocked BMP-9-pro-region complex activity, whereas the rest did not exhibit any inhibitory activity.

To measure relative affinities between BMP-9-pro-region complex and BMP/activin receptors, BLAcore analysis was performed using purified BMP-9-pro-region complex and receptor:Fc chimera immobilized on a BLAcore chip surface (Table II, column 1). BMP-9-pro-region complex did not bind the control flow cell. However, it did bind strongly to ALK-1 and BMPR-II. In addition, BMP-9 also binds weakly to ActR-IA, ActR-IIA, and ActR-IIB at different relative levels. Binding constants cannot be accurately derived from this study, as only one ligand concentration was applied to a high density chip, and it has been shown in other studies that dissociation constants vary based on the density of the fixed receptor and whether the ligand or the receptor was immobilized due to cooperative binding by the receptors (36, 37). However, by comparative analysis, it is clear that BMPR-II and ALK-1 have the highest affinity.

To further examine interactions between BMP-9 and ALK-1, BMP-9-pro-region complex was used in the absence or presence of various concentrations of soluble ALK-1 or BMP-IRA receptors in the C2C12 alkaline phosphatase and H4IIe/ME-SEAP reporter assays (Fig. 5). BMP-4 was included in the experiment as a control. ALK-1:Fc completely inhibited BMP-9-pro-region complex activity in both assays when given at >6-fold molar excess but showed no effect on BMP-4. As expected, BMPRIA:Fc inhibited BMP-4 activity in a dose-dependent manner, ranging from 1- to 6-fold molar excess but did not block activity of BMP-9-pro-region complex. Combining the results, our data demonstrate that ALK-1 can bind to purified BMP-9-pro-region complex and neutralize its biological activity in cell-based assays.

**DISCUSSION**

In the TGF-β superfamily, which consists of over 30 known ligands but only 7 type I and 5 type II receptors, overlapping specificities among the ligands and receptors are common. The characteristic structural scaffold of TGF-β family ligands is provided by the amino acids (boxed in gray in Fig. 2) forming the protein core or palm of the hand and in the receptors by the highly conserved set of disulfide bond-forming cysteines (39). This common structural framework likely provides the basis for overlapping specificities between ligands and receptors. Recent structural studies on the extracellular fragments of TGF-β receptors in complex with several related ligands have provided important insights on the roles of stoichiometry and affinity in determining the overall outcome of a ligand-receptor interaction (40–42). These studies highlighted the importance of the membrane in restricting the orientations of the two receptor subtypes, suggesting that the relative orientations and distances of the pair of high affinity receptors may influence the affinity of the ligand for the lower affinity receptors. Furthermore, as the first high affinity receptor binds to a ligand, it restricts the entropic freedom of the ligand, resulting in enhancement of binding to the second high affinity receptor to the complex (42, 43).

Crystallization of BMP-2 in complex with BMPRIA helped to identify the points of contact between a BMP ligand and type I receptor, and further studies identified the hydrogen bonds that determined binding affinity (44). In BMP-2, these are the amide and carbonyl of Leu$^{51}$ and the amide of Asp$^{55}$, and these residues are conserved in BMP-9. BMP-9, however, shows no affinity for BMPRIA in cell-based or BLAcore studies. Significant C-α r.m.s. deviations do occur between these residues, however, suggesting that main chain position in the pre-helix
loop may be an important determinant of binding affinity for type I receptors.

We have determined from the crystal structure of BMP-9 that there are few significant conformational differences between BMP-9 and BMP-7 at the binding sites for the type II receptors. These results are consistent with known promiscuity among type II receptors and our BIAcore data (indicating some binding affinity between BMP-9 and ActR-IIA) but do not exclude the possibility that the different splicing of exons encoding the 15-amino-acid linker between the extracellular domain and the transmembrane domain of each receptor may influence signaling efficiency.

The pro-regions of TGF-βs and GDF-8 remain associated with their ligands after secretion from the cell and have been found to be functionally inhibitory in vitro and in vivo (4–7). The mechanism for this inhibition is not known, although particular regions of the pro-region relevant for inhibition have been identified (5). The pro-region of BMP-9 also remains associated after secretion, but we have shown that both BMP-9 and BMP-9-pro-region complex were equally active in three cell-based assays covering a range of reported BMP-9 activities, including osteoinduction, proliferation, and gluconeogenesis (20, 21, 23). These assays demonstrated that the pro-region of BMP-9, unlike those of TGF-βs and GDF-8, does not appear to functionally inhibit BMP-9. It is not clear whether the BMP-9 pro-region does not block the binding sites for either receptor subtype or whether the pro-region is effectively competed off by one or both of the receptor subtypes. It is instead possible that the pro-region of BMP-9 may act to protect and stabilize BMP-9 in vivo.

BMP-9 shows a tissue expression profile largely restricted to the liver and has been shown to stimulate proliferation in hepatic cells (45), but its receptor has not been identified. ALK-1 is highly expressed in endothelial cells, and inactivating mutations of ALK-1 have been associated with hereditary hemorrhagic telangiectasia (26), a vascular pathology affecting cells were growth-arrested for 24 h in low serum medium and treated with either buffer or BMP-9, BMP-9-pro-region complex, or pro-region at the indicated concentrations (nM). After 4 days, the cells were lysed and alkaline phosphatase activity determined. B. H4IIe/ME-SEAP reporter assay. Serum-deprived reporter cells were treated with either buffer or BMP-9, BMP-9-pro-region complex, or pro-region at the indicated concentrations. SEAP activity in the conditioned medium was measured after a 48-hour incubation period. C. 3T3-L1 cell proliferation assay. 3T3-L1 cells were growth-arrested for 24 h in low serum medium and treated with either buffer or BMP-9, BMP-9-pro-region complex, or pro-region at the indicated concentrations. After 4 days, cell numbers were determined by Cell-Titer Glo assay. For all assays, the cells were treated in duplicate or triplicate wells and average ± S.D. for each treatment is shown. Data is normalized to vehicle-treated samples and presented as the signal from the BMP-9-treated sample divided by the signal from the vehicle-treated sample. AP, alkaline phosphatase.

**Table II.** Summary of inhibition studies with soluble receptors in C2C12 alkaline phosphatase assay and BIAcore analysis on binding of BMP-9-pro-region to soluble receptors

| Receptor       | Relative affinity of BMP-9-pro-region complex to receptor (BIAcore) | Inhibition of BMP-9 activity in AP assay | Inhibition of BMP-4 activity in AP assay |
|----------------|------------------------------------------------------------------|----------------------------------------|---------------------------------------|
| ALK-1          | 1801.8                                                           | +++                                    | -                                     |
| BMPR-II        | 1336.9                                                           | +                                      | -                                     |
| Activin RIIA   | 384.8                                                            | -                                      | -                                     |
| Activin RIIB   | 99.3                                                             | -                                      | -                                     |
| Activin RIA    | 39.9                                                             | -                                      | -                                     |
| ALK-7          | 1.6                                                              | -                                      | -                                     |
| BMPR-IB        | 4.7                                                              | -                                      | -                                     |
| BMPR-IA        | -3.9                                                             | -                                      | +++                                   |
| Activin RIB    | -10.1                                                            | -                                      | -                                     |

**Fig. 4.** Activities of BMP-9 and BMP-9-pro-region complex are similar in cell-based functional assays, whereas pro-region alone has no effect. A, C2C12 alkaline phosphatase assay: C2C12 alkaline phosphatase activity was measured in the presence of various concentrations of BMP-9, BMP-9-pro-region complex, or pro-region. B, ME-SEAP reporter assay: SEAP activity in the conditioned medium was measured after a 48-hour incubation period. C, 3T3-L1 cell proliferation assay: 3T3-L1 cells were treated with either buffer or BMP-9, BMP-9-pro-region complex, or pro-region at the indicated concentrations.
multiple organs. Transfection of constitutively active forms of ALK-1 has been shown to stimulate Smad-1 (46) and induce alkaline phosphatase expression in C2C12 cells (47). However, to date the ligand for the ALK1 receptor has not been clearly identified. In this report, we have provided the first evidence that ALK-1 is a strong candidate for a functional receptor for BMP-9. In cell-based assays employing two distinct cell types, a soluble ALK-1:Fc protein was able to potently and selectively block BMP-9-pro-region complex activity. BIAcore analysis indicates that BMP-9-pro-region complex binds to additional receptors to some degree, including BMPR-II, ActR-IIA, and ActR-IIB. Of these, only BMPR-II inhibited BMP-9-pro-region complex activity to any significant extent. The exact nature of the interactions of BMP-9 with ALK-1 and other receptors in

FIG. 5. Inhibition of BMP-9 complex activity by soluble ALK-1:Fc. A, C2C12 alkaline phosphatase assay. C2C12 cells were growth-arrested for 24 h in low serum medium. The cells were treated with either BMP-9-pro-region complex (5 nM) or BMP-4 (4.7 nM) alone or in the presence of the indicated -fold excess molar concentration of soluble ALK-1:Fc or BMP-RIA:Fc proteins. After 4 days, the cells were lysed and alkaline phosphatase activity determined. AP, alkaline phosphatase. B, H4IIe/ME-SEAP reporter assay. Serum-deprived reporter cells were treated with either BMP-9-pro-region complex (2 nM) or BMP-4 (0.1 nM) alone or in the presence of the indicated -fold excess molar concentration of soluble ALK-1:Fc or BMP-RIA:Fc proteins. SEAP activity in the conditioned medium was measured after a 48-h incubation period. All assays used chemiluminescent detection methods, and data were presented in relative light units (RLU). Experiments were performed with duplicate or triplicate wells, and average ± S.D. for each treatment is shown.
physiological functions and potential roles in diseases remains to be understood.

Acknowledgment—We thank W. Zhou for critical technical advice.

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J. Biol. Chem. 2005, 280:25111-25118.
doi: 10.1074/jbc.M503328200 originally published online April 25, 2005

Access the most updated version of this article at doi: 10.1074/jbc.M503328200

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