A Kinase Domain-truncated Type I Receptor Blocks Bone Morphogenetic Protein-2-induced Signal Transduction in C2C12 Myoblasts*

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Members of the transforming growth factor (TGF)-β superfamily bind the transmembrane serine/threonine kinase complex consisting of type I and type II receptors. Their intracellular signals are propagated via respective type I receptors. Bone morphogenetic protein (BMP)-2, a member of the TGF-β superfamily, induces ectopic bone formation when implanted into muscular tissues. Two type I receptors (BMPR-IA and BMPR-IB) have been identified for BMP-2. We have reported that BMP-2 inhibits the terminal differentiation of C2C12 myoblasts and converts their differentiation pathway into that of osteoblast lineage cells (Katagiri, T., Yamaguchi, A., Komaki, M., Abe, E., Takahashi, N., Ikeda, T., Rosen, V., Wozney, J. M., Fujisawa-Sehara, A., and Suda, T. (1994) J. Cell Biol. 127, 1755–1766). In the present study, we examined the involvement of functional BMP-2 type I receptors in signal transduction in C2C12 cells, which expressed mRNA for BMPR-IA, but not for BMPR-IB in Northern blotting. TGF-β type I receptor (TβR-I) mRNA was also expressed in C2C12 cells. Subclonal cell lines of C2C12 that stably expressed a kinase domain-truncated BMPR-IA (ΔBMPR-IA) differentiated into myosin heavy chain-expressing myotubes but not into alkaline phosphatase (ALP)-positive cells, even in the presence of BMP-2. In contrast, the differentiation of the ΔBMPR-IA-transfected C2C12 cells into myotubes was suppressed by TGF-β1, as in the parental C2C12 cells. BMP-2 did not efficiently suppress the mRNA expression of muscle-specific genes such as muscle creatine kinase, MyoD, and myogenin, nor did it induce the expression of ALP mRNA in the ΔBMPR-IA-transfected C2C12 cells. In contrast, TGF-β1 inhibited mRNA expression of the muscle-specific genes in those cells. When wild-type BMPR-IA was transiently transfected into the ΔBMPR-IA-transfected C2C12 cells, a number of ALP-positive cells appeared in the presence of BMP-2. Transfection of wild-type BMPR-IB or TβR-I failed to increase the number of ALP-positive cells. These results suggest that the BMP-2-induced signals, which inhibit myogenic differentiation and induce osteoblast differentiation, are transduced via BMPR-IA in C2C12 myoblasts.

Deminerlized bone matrix can induce ectopic bone formation when implanted into muscular tissues (1). Bone morphogenetic proteins (BMPs) purified and cloned from deminerlized bone matrix are the factors responsible for this ectopic bone formation (2–7). The deduced amino acid sequence of BMPs indicates that they are members of the transforming growth factor-β (TGF-β) superfamily (3, 6–8). Genetic studies show that BMPs and their related molecules are essential for normal skeletal development in vertebrates (9–11). When the BMP-5 or growth/differentiation factor (GDF)-5 gene was mutated in mice, they developed abnormal skeletons (12, 13). A genetic mutation of the human homologue of mouse GDF-5 was also identified in a patient with chondrodysplasia (14). Gene targeting showed that BMP-7-deficient mice had a defect in skeletal pattern formation besides those in the eye and kidney (15, 16).

Several lines of evidence indicate that the role of BMPs is not restricted to skeletal development but is expanded to diverse developmental events in animals (9–11). DPP, a homologue of human BMP-2 and BMP-4, acts as a morphogen in dorsal-ventral patterning of the Drosophila embryo. BMP-4 acts as a ventralizing factor in the Xenopus embryo. In BMP-4-deficient mice, the mesoderm did not differentiate during embryogenesis (17). It was also suggested that BMPs act as signaling molecules of epithelium-mesenchyme interaction in the development of various organs in vertebrates.

The intracellular signals of members of the TGF-β superfamily are transduced via transmembrane serine/threonine kinase receptors (9, 11, 18, 19). These are classified into two groups known as type I and type II receptors that are distinguishable by their amino acid sequences and functional features. Members of the TGF-β superfamily are associated with specific sets of the type I and type II receptors. Formation of a type I-type II receptor complex is required for ligand-induced signalings. Studies on the receptors for TGF-β have revealed the sequential mechanism of receptor activation (20). The TGF-β type II receptor (TβR-II) first binds to the ligand and then associates and phosphorylates TGF-β type I receptor (TβR-I). TβR-I is phosphorylated by TβR-II at serine and threonine residues in the GS domain, which is located upstream of the kinase do-

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† The abbreviations used are: BMP, bone morphogenetic protein; TGF-β, transforming growth factor-β; DPP, decapentaplegic; BMPR-I, -II, BMP receptor types I and II, respectively; TβR-I, -II, TGF-β receptor types I and II, respectively; ALP, alkaline phosphatase; GDF, growth/differentiation factor; ΔBMPR-IA, kinase domain-truncated BMPR-IA; DMEM, Dulbecco’s modified Eagle’s medium; FBS, fetal bovine serum; MCK, muscle creatine kinase; SDS, sodium dodecyl sulfate; MHC, myosin heavy chain; PAGE, polyacrylamide gel electrophoresis; kb, kilobases.
BMP-2-induced Signal Transduction

TGF-β signals are transduced by the BMP type I receptor like those of induced via BMPR-IA in C2C12 myoblasts.

Induced signals, namely, the inhibition of myogenic differentiating activity of BMP-4 in a dominant negative manner in the ever, the BMP-2 signaling pathway that regulates the myogenic induction of osteoblast differentiation of myoblasts. How-

BMPR-IB or T
 type BMPR-IA into kinase domain-truncated mouse BMPR-IA BMP-2 signaling was restored by transiently transfecting wild-type cells but also induces the differentiation of pluripotent fibroblastic cells into osteoblastic cells (29–32). To examine the molecular mechanism of ectopic bone formation induced by BMPs in muscular tissues, we established a model system using C2C12 myoblasts that reflects an early stage of osteoblast differentiation during bone formation in muscular tissues (33). In this model, BMP-2 not only inhibits the differentiation of C2C12 myoblasts into multinucleated myotubes but also induces the expression of typical osteoblast phenotypes such as alkaline phosphatase (ALP) activity, parathyroid hormone response, and osteocalcin production. TGF-β also inhibited terminal differentiation of C2C12 cells but did not induce any of the osteoblast phenotypes (33). These results suggested that some BMP-2-specific intracellular signalings are involved in the induction of osteoblast differentiation of myoblasts. However, the BMP-2 signaling pathway that regulates the myogenic and osteogenic differentiation of myoblasts is not clear.

A kinase domain-truncated BMPR-IA abolished the ventralizing activity of BMP-4 in a dominant negative manner in the Xenopus embryo (23, 34, 35). We therefore examined whether a dominant negative BMP type I receptor could inhibit the BMP-2-induced signalings in myoblasts. We report here that overexpression of the kinase domain-truncated BMPR-IA in C2C12 myoblasts blocks the BMP-2-induced inhibition of myogenic differentiation and induction of osteogenic differentiation. BMP-2 signaling was restored by transiently transfecting wild-type BMPR-IA into kinase domain-truncated mouse BMPR-IA (ΔBMPR-IA)-transfected C2C12 cells but not by transfecting BMPR-IB or TßR-1. These results suggest that the BMP-2-induced signals, namely, the inhibition of myogenic differentiation and the induction of osteoblast differentiation, are transduced via BMPR-IA in C2C12 myoblasts.

MATERIALS AND METHODS

BMP-2—Recombinant human BMP-2 was produced and purified from the conditioned media of Chinese hamster ovary cells as described (36).

Plasmide—The ΔBMPR-IA cDNA was generated by the standard PCR protocol using a set consisting of vector-derived upstream and mutated downstream primers. The latter had a stop codon at position 706 (encodes 188 amino acids) in the original mouse TßR-11 (23) followed by an EcoRI restriction site (5'-AAGAATTCACAGGCGCTTGGATACCT-3'). The kinase domain-truncated mouse BMPR-IB (ΔBMPR-IB) (24) cDNA was also generated by a similar method, and it encoded 147 amino acids. Mutations of those products were verified by DNA sequencing. These truncated receptor cDNAs were subcloned into the EcoRI site of the mammalian expression vector, pMIKHygB (provided Dr. K. Maruyama), which expresses an insert under the control of the SV40 promoter and has a hygromycin-resistant gene. Wild-type BMPR-IA, BMPR-IB, or TßR-1 (37) receptor cDNA was subcloned into pEF-BOS and expressed under the control of the human EF1α promoter (38). Constitutively active BMPR-IB (aBMPR-IB) was generated by a substitution of glutamine at 203 for aspartic acid by PCR. For the immunoprecipitation experiment, the wild-type BMPR-IB was tagged by introducing epitope sequences for human c-myc (EQKLISEEDE) into the carboxyl terminus.

Cell Culture—C2C12 myoblasts (39) were purchased from the American Type Culture Collection (Rockville, MD). Subclonal cell lines of C2C12 (see below) were maintained in Dulbecco’s modified Eagle’s medium (DMEM, Sigma) containing 15% fetal bovine serum (FBS; Life Technologies, Inc., Grand Island, NY) and antibiotics (100 units/ml penicillin and 100 µg/ml streptomycin) at 37 °C in a humidified atmosphere of 5% CO2 in air. To examine the effects of BMP-2 on muscle and osteoblast differentiation of the subclonal cell lines, C2C12 cells were inoculated at a density of 2 × 104 cells/cm2. After an overnight incubation, the medium was replaced with DMEM containing 5% FBS and 300 ng/ml BMP-2, and then the cells were cultured for an additional 3 days.

Transfection—To establish subclonal C2C12 cell lines that constitutively express ΔBMPR-IA or ΔBMPR-IB, C2C12 myoblasts were inoculated onto 100-mm dishes at a density of 5 × 104/dish 1 day before transfection by modified calcium phosphate precipitation according to an instruction manual (Stratagene, CA). In brief, C2C12 cells were incubated overnight with the calcium phosphate–DNA precipitates containing 10 µg of pMIKHygB, pMIKHygB/ΔBMPR-IA, or pMIKHygB/ΔBMPR-IB. They were split at a 1:20 ratio and selected for 10 days in the presence of 700 µg/ml hygromycin B (Wako Pure Chemical Industries, Osaka, Japan). Colonies were isolated with penicillin cups and passaged into stable cell lines. The subclonal cell lines, Vec-18 and Vec-19, ΔIA-12, ΔIA-14 and ΔIA-16 and ΔIB-2, and ΔIB-2, were established from cultures of myoblasts transfected with pMIKHygB, pMIKHygB/ARMPR-IA, and pMIKHygB/ΔBMPR-IB, respectively.

For rescue experiments, subclonal cells that stably expressed ΔBMPR-IA were transiently transfected with the wild-type type I receptor (BMPR-IA, BMPR-IB, or TßR-1) using DOTAP (N-[1-(2,3-dioleoyloxy)propyl]-N,N,N-trimethylammonium methyl sulfate) (Boehringer Mannheim, Germany). Cells were inoculated onto 48-well plates at a density of 2.5 × 104/well 1 day before transfection. The cells were then incubated for 10 h with OPTI-MEM (Life Technologies, Inc.) containing a mixture of the expression vector (pEF-BOS, pEF-BOS/BMPR-IA, pEF-BOS/BMPR-IB, pEF-BOS/TßR-1) and DOTAP according to an instruction manual. The cells were transferred to DMEM containing 15% FBS and 300 ng/ml BMP-2 and were cultured for an additional 3 days. Constitutively active BMPR-IB (pEF-BOS/ΔBMPR-IB) was also transfected into the subclonal cells that stably expressed ΔBMPR-IA by the same method described above, and the cells were cultured without BMP-2. Cells were fixed and histochemically stained for ALP as described below.

Northern Blot—Total cellular and poly (A)+ RNAs were extracted from cells using TRIZOL (Life Technologies, Inc.) and a QuickPrep Micro mRNA purification kit (Pharmacia P-L Biochemicals Inc., Milwaukee, WI), respectively. Twenty µg of total RNA or 2.5 µg of poly (A)+ RNA was resolved by electrophoresis in a 1.2% agarose-formaldehyde gel and transferred onto a Hybond-N membrane (Amersham International, Amersham, UK). The membrane was hybridized with [32P]-labeled cDNA probes for mouse BMPR-IA (23), rat BMPR-IB (24), mouse TßR-1 (37), mouse ALP (40), mouse osteocalcin (41), mouse MCK, mouse myogenin (42), and mouse myostatin (42). The hybridized probes were removed from the membrane by boiling in 0.2% sodium dodecyl sulfate (SDS) and then sequentially rehybridized with the respective probes.

Histochemical Analysis—To histochemically analyze the expression levels of myosin heavy chain (MHC) and ALP, MHC and ALP were double stained. Cells were fixed in 3.7% formaldehyde and then stained immunohistochemically for MHC using anti-MHC antibody (MF-20; Developmental Studies Hybridoma Bank, Iowa City, IA) (44) as described (33). Before color development, cultures were histochemically stained for ALP using naphthol AS-MX phosphate (Sigma) and fast blue BB salt (Sigma) (33). Cells were rinsed with phosphate-buffered saline, and then the reaction products of biotin-streptavidin were visualized with an AEC substrate kit (Histofine, Nichirei Co., Tokyo, Japan).

ALP Activity—ALP activity in the cell layer was measured as described (33). Cell layers were sonicated in 50 mM Tris-HCl, 1.0% Triton X-100, pH 7.5. ALP activity in the lysate was measured at 37 °C in a buffer containing 0.1 to 2-aminomethylene-1-propanol (Sigma) and 2 mM MgCl2, pH 10.5, for 30 min using p-nitrophenyl phosphate as the substrate. The enzyme activity was measured as micromoles of p-nitrophenol (p-NP) produced per minute per mg protein. The protein content was determined using a BCA protein assay kit (Pierce).

Receptor Affinity Labeling Assay—[125I]-labeled BMP-2 was prepared using chloramine-T as described previously (45). In short, cell layers were incubated for 2 h at 37 °C with 5 ng/ml 125I-BMP-2. Receptors were cross-linked to bound ligands with disuccinimidyl suberate (Pierce) and solubilized by sample buffer (25 mM Tris-HCl, pH 6.5, 5%
glycerol, 1% SDS, 1% 2-mercaptoethanol, 0.05% bromphenol blue) for SDS-polyacrylamide gel electrophoresis (PAGE). Aliquots were subjected to SDS-PAGE. The autoradiogram was analyzed using a BAS 2000 Bioimaging Analyzer (Fuji Photo Film, Tokyo, Japan).

For the immunoprecipitation of receptor proteins, affinity-labeled cells were lysed for 1 h in TNE buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1 mM EDTA) containing 0.5% Triton X-100 and 10 μg/ml aprotinin. For the isolation of receptor complexes, cell extracts were clarified by centrifugation and precipitated with e-myc antibody (9E10; BabCO, Richmond, CA) followed by binding to protein G-Sepharose (Zymed Laboratories, San Francisco, CA). Immunoprecipitates were washed five times with TNE buffer containing 0.1% Triton X-100 and subjected to SDS-PAGE and autoradiography.

RESULTS

C2C12 Myoblasts Express BMPR-IA but Not BMPR-IB—First, we examined the mRNA levels of the two BMP type I receptors in C2C12 myoblasts by Northern blotting (Fig. 1). C2C12 cells expressed two major forms of BMPR-IA mRNAs of –6 and 3.6 kb. C2C12 cells also expressed TβR-I mRNA of –6 kb. However, a BMPR-IB transcript was not detected in C2C12 cells by Northern blotting.

Transfection of a Kinase Domain-truncated BMPR-IA into C2C12 Cells Specifically Blocks BMP-2 Signals—To examine the role of BMPR-IA on the BMP-2 signaling pathways in C2C12 cells, we constructed ΔBMPR-IA and ΔBMPR-IB expression vectors and stably transfected them into C2C12 cells. The subclonal cell lines, ΔIA-12, ΔIA-14 and ΔIA-16, ΔIB-2 and ΔIB-12, and Vec-16 and Vec-19, were established from C2C12 cells transfected with the empty vector (Vec), the ΔBMPR-IA expression vector (ΔIA), and the ΔBMPR-IB expression vector (ΔIB). Cells were cultured in DMEM containing 5% FBS in the presence of 300 or 1000 ng/ml BMP-2. After culture for 3 days, myotube formation (A) and ALP activity (B) were measured as described under “Materials and Methods.” Data are the means ± S.E. of triplicate cultures.

Fig. 3 shows the histochemical changes induced by BMP-2 and TGF-β1 in Vec-16, ΔIA-12, and ΔIB-12 cells. Elongated MHC-positive cells appeared in all the cultures in the absence of BMP-2 and TGF-β1 (Fig. 3, a, d, and g). In Vec-16 and ΔIB-12 cells, BMP-2 inhibited the formation of MHC-positive myotubes and induced ALP-positive osteoblast-like cells (Fig. 3, b and h). In contrast, ΔIA-12 cells differentiated into MHC-positive, but not into ALP-positive cells, even in the presence of 300 ng/ml BMP-2 (Fig. 3e). When these empty vector-, ΔBMPR-IA-, and ΔBMPR-IB-transfected cells were cultured with TGF-β1, differentiation into MHC-positive cells was greatly suppressed in all the cultures examined (Fig. 3, c, f, and i).

We examined the expression of receptor proteins in the subclones transfected with ΔBMPR-IA (ΔIA-12), ΔBMPR-IB (ΔIB-12), or the empty vector (Vec-16) by the affinity labeling assay using [125I]-BMP-2. Several [125I]-BMP-2-cross-linked proteins with lower and higher molecular masses were detected in ΔIA-12 cells, which disappeared by adding cold BMP-2 (Fig. 4, lanes 1 and 2). However, no appreciable signals of the proteins labeled with [125I]-BMP-2 were observed in ΔIB-12 (Fig. 4, lane 3) and Vec-16 cells (lane 5).

The Effects of BMP-2 and TGF-β1 on the Expression of mRNAs of Muscle- and Osteoblast-specific Phenotypes in the C2C12 Cells Transfected with ΔBMPR-IA and ΔBMPR-IB—To further determine the role of BMPR-IA on BMP-2 signalings in
C2C12 cells, we examined mRNA expression of BMP-2 and TGF-β receptors and muscle- and osteoblast-specific phenotypes by Northern blotting in Vec-16, ΔIA-12, and ΔIB-12 cells. When the mRNA levels of BMPR-IA were analyzed using an extracellular domain probe, endogenous BMPR-IA mRNA was detected in all the cell lines at an equivalent level (Fig. 5). Truncated BMPR-IA mRNA was also detected in ΔIA-12 cells (Fig. 5). A kinase domain probe of BMPR-IA was hybridized with the endogenous mRNA but not with the truncated BMPR-IA mRNA (data not shown). Truncated BMPR-IB mRNA was detected only in ΔIB-12 cells, but endogenous BMPR-IB mRNA was not detected in any of these cell lines. All three cell lines expressed TβR-I mRNA, irrespective of the presence and absence of BMP-2 or TGF-β1. In Vec-16 cells, BMP-2 suppressed the expression of muscle-specific mRNAs such as MCK and myogenin and induced osteocalcin mRNA, a specific marker for osteoblasts. These muscle-specific mRNAs were expressed in ΔIA-12 cells even in the presence of BMP-2. Although myogenin mRNA was weakly detected in BMP-2-treated ΔIB-12 cells, BMP-2 completely suppressed the expression of MCK mRNA and strongly induced osteocalcin mRNA. However, TGF-β1 suppressed the expression of the muscle-specific mRNAs in all cell lines tested. ΔIA-12 cells did not express osteocalcin mRNA, irrespective of the presence or absence of BMP-2.

The Impaired ALP-inducing Activity of BMP-2 in ΔBMPR-IA-transfected C2C12 Cells Is Rescued by the Overexpression of Wild-type BMPR-IA—We performed a rescue experiment to examine whether the blocked signals of BMP-2 in ΔBMPR-IA-transfected cells are rescued by the overexpression of wild-type type I receptors. The wild-type BMPR-IA, BMPR-IB, and TβR-I were transiently transfected into ΔIA-12 and ΔIA-14 cells, and they were incubated with or without BMP-2. The BMP-2 signals were evaluated by the induction of ALP. A number of ALP-positive cells appeared when the wild-type BMPR-IA was overexpressed in ΔIA-12 and ΔIA-14 cells (Fig. 6, a, c, d, e, g, and h). However, transfection with either an empty vector or wild-type BMPR-IB or TβR-I failed to increase the number of ALP-positive cells in ΔIA-12 and ΔIA-14 cells (Fig. 6, b and f). ALP-positive cells did not appear in the absence of BMP-2 (data not shown). To confirm that C2C12 cells are capable of expressing exogenously introduced wild-type BMPR-IB proteins on the cell surface, we examined the binding ability of exogenously intro-

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**Fig. 3.** Histochemical changes induced by BMP-2 and TGF-β1 in the C2C12 cells transfected with ΔBMPR-IA. The cell lines, Vec-16 (a-c), ΔIA-12 (d and f), and ΔIB-12 (g-i), were cultured in DMEM containing 5% FBS in the absence (a, d, and g) and presence of 300 ng/ml BMP-2 (b, e, and h) or 10 ng/ml TGF-β1 (c, f, and i). After culture for 3 days, the cells were fixed and double stained for MHC (red) and ALP (blue). Scale bars, 100 μm.

**Fig. 4.** Affinity labeling of BMP receptors in C2C12 cells expressing truncated type I receptors. The cell lines, ΔIA-12, ΔIB-12, and Vec-16, were cultured in DMEM containing 15% FBS and affinity-labeled with 5 ng/ml 125I-BMP-2 in the presence (+) and absence (−) of 200 ng/ml cold BMP-2, as described under "Materials and Methods." Aliquots of the whole cell lysates were resolved by 7.5% SDS-PAGE and applied to autoradiography. Positions of receptor proteins and molecular markers (in kilodaltons, kDa) are indicated by the arrows on the left and bars on the right, respectively.

**Fig. 5.** The effects of BMP-2 and TGF-β1 on the expression of mRNAs in the C2C12 cells transfected with ΔBMPR-IA. The cell lines, Vec-16, ΔIA-12, and ΔIB-12, were cultured in DMEM containing 5% FBS in the absence (C) and presence of 300 ng/ml BMP-2 (B) or 10 ng/ml TGF-β1 (T). Total RNA from each culture (20 μg/lane) was resolved in a 1.2% formaldehyde-agarose gel and sequentially hybridized with the respective cDNA probes of BMPR-IA, BMPR-IB, TβR-I, muscle creatine kinase (MCK), osteocalcin, and myogenin. Arrows indicate exogenous ΔBMPR-IA and ΔBMPR-IB mRNAs.
BMPR-IA (siently transfected with the wild-type contrast, the differentiation into myotubes of the truncated like ALP-positive cells, even in the presence of BMP-2. In differentiated into MHC-positive myotubes but not into osteoblast- BMPR-IA was overexpressed in C2C12 myoblasts, they differ- entiation pathway into osteoblast lineage cells (33). This model suggests that when the type I receptor is propagated via their own type I receptors. Two type I recep- tors, BMPR-IA and BMPR-IB, have been identified for BMP-2/BMP-4. C2C12 cells expressed BMPR-IA mRNA but not BMPR-IB mRNA as detectable levels by Northern blotting. BMPR-IB mRNA was barely detected by reverse transcription-PCR with excessive amplification. Moreover, reducing the mRNA expression of TGF-β type I receptor causes resistance to the growth suppression induced by TGF-β in mink lung epithe-elial cells (46). This suggests that when the type I receptor is expressed at a low level, ligand-induced signals cannot be transduced. The present study suggests that the BMP-2 signals are mainly transduced via BMPR-IA but not via BMPR-IB in C2C12 cells because BMPR-IB was expressed at an extremely low level in those cells. To elucidate the involvement of BMPR-IA in the BMP-2 signaling in C2C12 cells, a kinase domain-truncated BMPR-IA was constructed and stably trans- fected into C2C12 cells. This truncated BMPR-IA acts as a dominante negative receptor for BMP-4 signaling in the Xenopus embryo (23, 34, 35). When the kinase domain-truncated BMPR-IA was overexpressed in C2C12 myoblasts, they differ- entiated into MHC-positive myotubes but not into osteoblast- like ALP-positive cells, even in the presence of BMP-2. In contrast, the differentiation into myotubes of the truncated BMPR-IB proteins in ΔIA-12 cells by the affinity cross-linking and immunoprecipitation using 125I-BMP-2. A weak but significant band corresponding to the wild-type BMPR-IB was observed in the immunoprecipitates of ΔIA-12 cells transfected with the wild-type BMPR-IB (Fig. 7a). To further examine the presence of the signaling pathway from BMPR-IB in C2C12 cells, we transiently transfected the constitutively active BMPR-IB into ΔIA-12 cells. A number of ALP-positive cells appeared in the culture even in the absence of BMP-2 (Fig. 7b).

**DISCUSSION**

In this study, we examined the involvement of the functional type I receptor for BMP-2 in C2C12 myoblasts in the molecular mechanism of osteoblast differentiation induced by BMPs. We reported that BMP-2 inhibits the differentiation of C2C12 cells into multinucleated myotubes and converted their differentiation pathway into osteoblast lineage cells (33). This model appears to reflect an early stage of osteoblast differentiation from non-osteogenic cells during BMP-induced bone formation in muscular tissues.

The intracellular signals of the TGF-β superfamily appear to be propagated via their own type I receptors. Two type I recep- tors, BMPR-IA and BMPR-IB, have been identified for BMP-2/BMP-4. C2C12 cells expressed BMPR-IA mRNA but not BMPR-IB mRNA as detectable levels by Northern blotting. BMPR-IB mRNA was barely detected by reverse transcription-PCR with excessive amplification. Moreover, reducing the mRNA expression of TGF-β type I receptor causes resistance to the growth suppression induced by TGF-β in mink lung epithelial cells (46). This suggests that when the type I receptor is expressed at a low level, ligand-induced signals cannot be transduced. The present study suggests that the BMP-2 signals are mainly transduced via BMPR-IA but not via BMPR-IB in C2C12 cells because BMPR-IB was expressed at an extremely low level in those cells. To elucidate the involvement of BMPR-IA in the BMP-2 signaling in C2C12 cells, a kinase domain-truncated BMPR-IA was constructed and stably trans- fected into C2C12 cells. This truncated BMPR-IA acts as a dominante negative receptor for BMP-4 signaling in the Xenopus embryo (23, 34, 35). When the kinase domain-truncated BMPR-IA was overexpressed in C2C12 myoblasts, they differ- entiated into MHC-positive myotubes but not into osteoblast- like ALP-positive cells, even in the presence of BMP-2. In contrast, the differentiation into myotubes of the truncated BMPR-IB-transfected cells was inhibited by TGF-β1. Moreover, the impaired ALP-inducing activity of BMP-2 was res- cued when the wild-type BMPR-IA was overexpressed in the cells expressing truncated BMPR-IA. These results suggest that BMPR-IA transduces two distinct signals specific for BMP-2 in C2C12 myoblasts, namely, the inhibition of myogenic differentiation and the induction of osteoblast differentiation. It is believed that the kinase domain-truncated type I recep- tor for the TGF-β superfamily inhibits the ligand-induced signals through competition during the formation of the complex of endogenous type I and type II receptors. C2C12 cells expressed BMPR-II mRNA. This suggests that the BMP-2 signals are transduced by the complex of BMPR-IA and BMPR-II in these cells. It was reported that both BMPR-IA and BMPR-IB formed complexes with BMPR-II or DAF-4, a type II receptor for a homologue of BMP-2/BMP-4 in C. elegans, in a ligand-dependent manner when overexpressed together on the surface of COS cells (21, 22, 25–27). In contrast to the truncated BMPR-IA, the kinase domain-truncated BMPR-IB did

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not inhibit the BMP-2 signaling in C2C12 cells even if its mRNA was abundantly expressed. Moreover, the truncated BMPR-IB was not cross-linked with 125I-BMP-2. These results indicate that the truncated BMPR-IB does not bind BMP-2 with a high affinity in C2C12 cells. Interestingly, Nohno et al. (27) reported that a truncated BMPR-II (BRK-3) did not efficiently cross-link to 125I-BMP-4 when expressed alone in COS cells. However, coexpression of the truncated BMPR-II with the type I receptor enhanced the binding to 125I-BMP-4. Although we could not detect endogenous type I nor type II BMP receptors in ΔIB-12 and Vec-16 cells by cross-linking using 125I-BMP-2, these endogenous receptors were detected in the truncated BMPR-IA-overexpressed cells (Fig. 4). Taking together, these results suggest that the endogenous receptors require sufficient amount of type I and type II receptors to bind ligands with a high affinity.

In the present study, wild-type BMPR-IB failed to rescue the impaired ALP-inducing activity of BMP-2 in C2C12 cells transfected with truncated BMPR-IA. The reason for this is not clear, but there are at least two possible explanations. First, the downstream signaling pathways of BMPR-IA and BMPR-IB may be different, and C2C12 cells lack the pathway for BMPR-IB. To test this notion, we examined the effects of a constitutively active mutant BMPR-IB on the induction of ALP activity. Wieser et al. (47) and Attisano et al. (48) reported that similar mutant receptors of TβR-I and activin type IIIB constitutively activate their kinases and transduce the ligand-induced signals in the absence of the ligands and appropriate type II receptors. Transfection of constitutively active BMPR-IB into truncated BMPR-IA-expressing C2C12 cells induced ALP activity even in the absence of BMP-2. Moreover, the stable transfection of constitutively active BMPR-IB into C2C12 cells inhibited their differentiation into the absence of BMP-2 (49). These results clearly indicate that C2C12 cells have a functional downstream signaling pathway for BMPR-IB.

Secondly, BMPR-IB may not be able to associate with BMPR-II (or other type II receptors that are associated with BMPR-IA), or BMPR-IB may not be activated by BMPR-II in C2C12 cells. Although C2C12 cells expressed BMPR-II mRNA, it is not clear whether BMPR-IA and BMPR-IB share the same BMPR-II that was expressed at the physiological level in C2C12 cells. If BMPR-IB associated with the same type II receptor for BMPR-IA in C2C12 cells, a kinase domain-truncated BMPR-IB should have inhibited the BMP-2-induced signals in these cells. The affinity binding assay followed by immunoprecipitation indicates that the wild-type BMPR-IB was expressed on the cell surface and bound BMP-2 in ΔIBMPR-IA-expressing C2C12 cells (Fig. 7a). Moreover, we recently found that the wild-type BMPR-IB-overexpressed C2C12 cells responded to another member of the BMP subfamily. These results suggest that the wild-type BMPR-IB can transduce BMP signaling when its appropriate ligand is present. Interestingly, Liu et al. (26) reported that BMPR-IB-BMPR-II complex could bind BMP-2 and BMP-7 although BMP-2 could not activate the transcription of reporter genes in COS cells. These results suggest that BMPR-IB is associated with a different type II receptor from that for BMPR-IA. Further studies are needed to prove this hypothesis.

At present, a mediator of the osteoblast differentiation-inducing signals of BMP-2 that acts at a downstream site of BMP type I receptor has not been elucidated. Genetic studies in Drosophila have identified Mothers against dpp (Mad), a gene that acts downstream of the signals of DPP, a Drosophila homologue of mammalian BMP-2/BMP-4 (50, 51). Mad-related proteins mimic the BMP-4 and activin effects in Xenopus (52, 53). Recently, a human homologue of Mad was identified as a possible signaling molecule for BMP-2 and BMP-4 in mammals (53, 54). The Mad-related protein was expressed in the cytoplasm and accumulated into the nucleus by the BMP-2/BMP-4 signals that were transduced by BMPR-IA or BMPR-IB (53, 54). These results suggest that Mad and/or its related proteins also mediate the osteoblast differentiation-inducing signals of BMP-2 via the activated type I receptor in some cells, including C2C12 myoblasts. Further studies are necessary to confirm this notion.

In conclusion, both BMP-2-induced signals, namely, the inhibition of myogenic differentiation and the induction of osteoblast differentiation, are transduced via BMPR-IA in C2C12 myoblasts. The downstream signaling pathway of BMPR-IA is involved in the ectopic bone formation induced by BMP-2 in muscular tissues. This in vitro model of C2C12 myoblasts is a useful system in which to investigate the molecular mechanism of not only osteoblast differentiation, but also of the BMP-2 signaling pathways.

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