Identification of Type I and Type II Serine/Threonine Kinase Receptors for Growth/Differentiation Factor-5*

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Growth/differentiation factor-5 (GDF-5) is a member of the bone morphogenetic protein (BMP) family, which plays an important role in bone development in vivo. Mutations in the GDF-5 gene result in brachypodism in mice and Hunter-Thompson type chondrodysplasia in human. BMPs transduce their effects through binding to two different types of serine/threonine kinase receptors, type I and type II. However, binding abilities appear to be different among the members of the BMP family. BMP-4 binds to two different type I receptors, BMP receptors type IA (BMPR-IA) and type IB (BMPR-IB), and a type II receptor, BMP receptor type II (BMPR-II). In addition to these receptors, osteogenic protein-1 (OP-1, also known as BMP-7) binds to activin type I receptor (ActR-I) as well as activin type II receptors (ActR-II and ActR-IIB). Here we investigate the binding and signaling properties of GDF-5 through type I and type II receptors. GDF-5 induced alkaline phosphatase activity in a rat osteoprogenitor-like cell line, ROB-C26. 125I-GDF-5 bound to BMPR-IB and BMPR-II but not to BMPR-IA in ROB-C26 cells and other nontransfected cell lines. Analysis using COS-1 cells transfected with the receptor cDNAs revealed that GDF-5 bound to BMPR-IB but not to the other type I receptors when expressed alone. When COS-1 cells were transfected with type II receptor cDNAs, GDF-5 bound to ActR-II, ActR-IIB, and BMPR-II but not to transforming growth factor-β type II receptor. In the presence of type II receptors, GDF-5 bound to different sets of type I receptors, but the binding was most efficient to BMPR-IB compared with the other type I receptors. Moreover, a transcriptional activation signal was efficiently transduced by BMPR-IB in the presence of BMPR-II or ActR-II after stimulation by GDF-5. These results suggest that BMPR-IB mediates certain signals for GDF-5 after forming the heteromeric complex with BMPR-II or ActR-II.

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1 The abbreviations used are: GDF, growth/differentiation factor; CDMP, cartilage-derived morphogenetic protein; BMP, bone morphogenetic protein; TGF-β, transforming growth factor-β; DPP, decapentaplegic gene product; OP, osteogenic protein; ALK, activin receptor-like kinase; TβR, TGF-β receptor; ActR, activin receptor; BMPR, BMP receptor; FBS, fetal bovine serum; ALP, alkaline phosphatase; NTA, nitrilotriacetic acid.
tors of about 50–55 kDa and type II receptors of more than 70 kDa (20–22). A series of receptor serine/threonine kinases, termed activin-like kinase (ALK)-1 to -6, was previously identified to constitute a type I receptor family, including a TGF-β type I receptor (TβR-I/ALK-5), two activin type I receptors (ActR-I/ALK-2 and ActR-IIB/ALK-4), and two BMP type I receptors (BMPR-IA/ALK-3 and BMPR-IB/ALK-6) (23–29). Type II receptors for activin (ActR-II and ActR-IIB) (30–32), for TGF-β (TβR-II) (33), and for BMPs (BMPR-II) (34–37) have been identified in mammals. In the TGF-β and activin receptor systems, ligand binds first to its specific type I receptor, and the complex of ligand and type II receptor is then recognized by type I receptor. Upon formation of the hetero-

meric receptor complex, type I receptor is phosphorylated by type II receptor, and subsequent activation of the catalytic activity of type I receptor kinase is essential for signaling (38, 39).

BMP-4 binds to BMPR-IA and BMPR-IB efficiently (27, 28, 40, 41) in the presence of DAF-4, a type II receptor in Caenorhabditis elegans (42), whereas OP-1/BMP-7 binds to BMPR-IB and less efficiently to BMPR-IA (27). OP-1/BMP-7, but not BMP-4, can also bind to one of the activin type I receptors, ActR-I, in the presence of DAF-4 (27). In addition, OP-1/BMP-7 was recently shown to bind ActR-II and ActR-IIB and mediate certain activin-like effects through the ActR-II-ActR-1 complex (43). We have recently shown that AK-1 (also termed TGF-β superfamily receptor type I or R3) mediates certain signals after stimulation by OP-1/BMP-7. Human BMPR-II was recently cloned, and it was shown that BMP-2, BMP-4, and OP-1/BMP-7 bound to BMPR-IA and transduced signals in combination with certain type I receptors after forming hetero-

meric complexes (35–37). In contrast to the TGF-β and activin receptors, BMP type I and type II receptors bind ligands independently, but binding affinity is up-regulated in the presence of both receptor types. The ligand-receptor interactions of BMPs in mammals are remarkably similar to those observed with the DPP receptor system in Drosophila (44, 45). However, the precise signaling mechanism of the BMP/DPP receptor systems remains unknown.

We investigated the biological effect of GDF-5 on osteopro-
genitor-like cell lines and identified type I and type II receptors for GDF-5; BMPR-IA and BMPR-IIB, but not BMPR-IAA, bound GDF-5 in ROB-C26 cells and other cell types. Moreover, we show here that GDF-5 transduces its signal through hetero-

meric complexes of BMPR-IB and various type II receptors.

**EXPERIMENTAL PROCEDURES**

**Cell Culture—** Mink lung epithelial cells (Mv1Lu) and COS-1 cells were obtained from American Type Culture Collection (Rockville, MD). Chemically mutagenized Mv1Lu cell line (R mutant, clone 4-2) (46) and U-1240 MG human glioblastoma cells (47) were obtained from M. Laio (University of Helsinki, Finland) and J. Massagüé (Memorial Sloan-Kettering Cancer Center, New York), and Bengt Westermark (University of Helsinki, Finland) and J. Massague (Memorial Sloan-Kettering Cancer Center, New York). Human fibroblasts (R mutant Mv1Lu cells) were co-

transfected with p3TP-Lux promoter-reporter construct (25, 54) with plasmids containing the type I or type II receptor cDNAs as described (48). Transient transfection plasmids encoding the type I receptors were previously described (26, 27). ActR-II cDNA was a gift from L. S. Mathews and W. W. Vale (Salk Institute, San Diego, CA). ActR-IIB DNA, BMPR-II cDNA construct and p3TP-Lux promoter-reporter construct were obtained from J. Massagüé. For transient transfection, cDNAs for type I or type II receptors subcloned into pSV5d (52), pcDNA1, pcDNA3 (Invitrogen), or pCMV5 (53) expression vectors were used. These cDNAs and p3TP-Lux promoter-reporter construct (1 µg of each) were transfected into COS-1 or R mutant Mv1Lu cells by the transfection kit of eukaryotic cells (TfxTM-50, Promega), following the manufacturer’s protocol. One or two days after transfection, the cells were used for affinity cross-linking and immunoprecipitation studies or transcriptional response assay.

Binding, Affinity Cross-linking, and Isolation of the Cross-linked Complexes—Recombinant human BMP-2 and GDF-5 were iodinated according to the chloramine-T method as described (26). Cells were incubated on ice for 2–3 h with 0.2–0.5 mCi of 125I-labeled ligands in the presence or absence of unlabeled ligands in a binding buffer (phosphate-buffered saline containing 0.1 mM CaCl2, 0.49 mM MgCl2, and 1 mM bovine serum albumin). After incubation, the cells were washed with the binding buffer without bovine serum albumin, and cross-linking was performed in the same binding buffer (32.5 mM disuccinimidyl suberate (Pierce) and 1 mM of bis(sulfosuccinimidyl) suberate (Pierce) for 15 min on ice. The cells were washed once with a buffer containing 10 mM Tris-HCl, pH 7.4, 1 mM EDTA, 10% glycerol, and 0.3 mM phenylmethylsulfonyl fluoride (Sigma) and lysed for 20 min in lysis buffer (20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 10 mM EDTA, 1% Triton X-100, 1% sodium deoxycholate) containing 1.5% Trasylol (Bayer) and 1 mM phenylmethylsulfonyl fluoride (Sigma) and dithiothreitol (Sigma). Cross-linked materials were then incubated with anti-sera for 45 min at 4°C. Immune complexes were bound to protein A-Sepharose (Kabi-Pharma-

dia) for 30 min at 4°C, washed once with a buffer containing 20 mM Tris-HCl, pH 7.5, 500 mM NaCl, 1% Triton X-100, 0.2% SDS, followed by one wash in distilled water. For isolation of BMPR-II cDNAs, complexes were extracted with a buffer containing 50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.5% Triton X-100, and protease inhibitors. Cell extracts were clarified by centrifugation and incubated with Ni2+-NTA-agarose (Qiagen) for 1 h at 4°C in the presence of 20 µM imidazole. Beads were rinsed briefly once with the same buffer. The immune complexes or complexes isolated by Ni2+-NTA-agarose were eluted by boiling for 3 min in SDS sample buffer (100 mM Tris-HCl, pH 8.8, 0.01% bromophenol blue, 36% glycerol, 4% SDS) containing 10% dithiothreitol and analyzed by SDS-7% polyacrylamide gel electrophoresis. The gels were fixed, dried, and subjected to the analysis using a Fujifilm BAS 2000 Bio-Imaging Analyzer (Fuji Photo Film).

Transcriptional Response Assay—R mutant Mv1Lu cells were co-

transfected with p3TP-Lux promoter-reporter construct (25, 54) with plasmids containing the type I or type II receptor cDNAs as described above. One day after transfection, cells were starved in Dulbecco’s modified Eagle’s medium containing 0.2% FBS for 6 h and then exposed to 300 ng/mL of GDF-5 for 24 h. Luciferase activity in the cell lysate was measured using the luciferase assay system (Toyo Ink) according to the manufacturer’s protocol and a luminometer (AutoLumat LB953; EG&G Berthold).

**Identification of GDF-5 Receptors**

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RESULTS

ALP Activity—GDF-5 stimulated mesenchyme aggregation and chondrogenesis in rat limb bud cells in vitro and induced ectopic cartilage and bone formation in mice tissues of rodents in vivo (14). However, several osteoblastic cell lines, such as MC3T3-E1 cells, did not efficiently respond to GDF-5 as measured by ALP activity in contrast to the effects of other BMPs. To identify the signaling receptors for GDF-5, we first attempted to find cell lines that respond to GDF-5 using the enzyme histochemical study of ALP activity. In ROB-C26 rat osteoprogenitor-like cell line, GDF-5 increased the number of ALP-positive cells at 300 ng/ml (Fig. 1A). However, most cell lines, including MC3T3-E1 mouse osteoblastic cells, ROS17/2.8 rat osteosarcoma cells, and C2C12 mouse myoblastic cells, had no significant increase in ALP activity by the treatment with GDF-5 (data not shown). Fig. 1B shows the dose-dependent effect of GDF-5 on ROB-C26 cells treated for 6 days. ALP activity was induced by GDF-5 in a dose-dependent manner.

Identification of GDF-5 Receptors in Nontransfected Cell Lines—In order to investigate which serine/threonine kinase receptors act as type I and type II receptors for GDF-5, the ROB-C26 cells were tested for the binding of GDF-5. The cells were affinity-labeled using 125I-GDF-5, and the cross-linked complexes were analyzed by immunoprecipitation using the antiserum against each of type I and type II receptors, followed by SDS-gel electrophoresis under reducing conditions. Cross-linked complexes of 80–90 kDa could be immunoprecipitated by the antiserum to BMPR-IB (Fig. 2A). A high molecular mass complex of 150–200 kDa, which may represent a type II receptor complex, was co-immunoprecipitated by the BMPR-IB antiserum. The type II receptor complex could be immunoprecipitated by the BMPR-II antiserum, and co-immunoprecipitation of the type I receptor complex was also seen (Fig. 2A). Immunoprecipitation by the BMPR-II antiserum was less efficient than that by the BMPR-IB antiserum, which may be due to a poor affinity of the BMPR-II antiserum (36). Weak bands could be seen after immunoprecipitation by the antisera against ALK-1, BMPR-IA, and TβR-II (Fig. 2A), but these were not reproducible.

Binding of 125I-BMP-2 was also tested in the ROB-C26 cells (Fig. 2B). Although 125I-BMP-2 is known to bind BMPR-IA and BMPR-IB as well as BMPR-II in other systems, binding to only BMPR-IB and BMPR-II could be seen in this cell type, suggesting that this cell line predominantly expresses BMPR-IB rather than BMPR-IA.

The binding of 125I-GDF-5 and 125I-BMP-2 to BMPR-IB was competed with unlabeled GDF-5 and also with BMP-2 (Fig. 2C).

To identify the endogenous receptors for GDF-5 in other cell types, we tested some other cell lines for the binding of GDF-5. In the U1240 MG glioblastoma cell line and the Mv1Lu mink lung epithelial cell line, BMPR-IB could bind GDF-5 (Fig. 3). In contrast, 125I-BMP-2-cross-linked complexes to Mv1Lu were immunoprecipitated by BMPR-IA (data not shown). In most cell types investigated, including ATDC5 chondroblastic cells, MC3T3-E1 mouse osteoblastic cells, ROS17/2.8 rat osteosarcoma cells, BEC bovine endothelial cells, and C2C12 mouse myoblastic cells, binding of GDF-5 was not clear (data not shown).

Binding of GDF-5 to Type I and Type II Receptors Expressed in COS-1 Cells—In order to further investigate the type I and type II receptors for GDF-5, binding was tested using COS-1 cells transfected with the cDNAs for serine/threonine kinase receptors. For the transfection of BMPR-II cDNA, a C-terminally truncated form of BMPR-II(His), which encodes 530 amino acid residues with a hexahistidine tag in its C terminus and, therefore, forms about 100 kDa of cross-linked complexes (35), was used. The cross-linked complexes were precipitated using the specific antisera or Ni²⁺-NTA agarose beads.

When singly transfected, we could observe binding of GDF-5 only to BMPR-IB among six type I receptors. Among different type II receptors, ActR-II, ActR-IB1, and BMPR-II(His) bound GDF-5 (Fig. 4). GDF-5 did not bind well to other serinethreo-
nine kinase receptors, including BMPR-IA, ActR-I (Fig. 4), and DAF-4, a BMP type II receptor in C. elegans (data not shown).

When type I receptor cDNAs were co-transfected with the BMPR-IIHis, ActR-II, or ActR-IIB1 cDNA, GDF-5 bound different sets of type I receptors. In the presence of BMPR-IIHis, GDF-5 bound efficiently to BMPR-IB, but not to the other type I receptors (Fig. 5A). BMPR-IB as well as BMPR-II bands could be seen when the cross-linked complexes were immunoprecipitated by the BMPR-IB antiserum. When isolated with Ni²⁺-NTA agarose beads, co-precipitation of the BMPR-IB complex could also be observed (Fig. 5A). Binding to BMPR-IB was up-regulated in the presence of BMPR-IIHis, compared with its absence (data not shown). In the presence of ActR-II, GDF-5 bound most efficiently to BMPR-IB (Fig. 5B). Weak binding of GDF-5 was also seen to BMPR-IA in the presence of ActR-II (Fig. 5B). In the presence of ActR-IIB1, GDF-5 bound efficiently to BMPR-IB, but also to ActR-I and to BMPR-IA very weakly (Fig. 5C). Thus, the binding of GDF-5 is most efficient to BMPR-IB compared with the other type I receptors, and weak binding to BMPR-IA and ActR-I is observed in the presence of different type II receptors. When COS-1 cells were co-transfected with BMPR-IB and ActR-IIB1 cDNAs, the BMPR-IB complex could not be immunoprecipitated with the type II receptor antiserum (Fig. 5C), suggesting that BMPR-IB forms a heteromeric complex with BMPR-II and ActR-II upon GDF-5 binding but less efficiently with ActR-IIB1.

Signaling Activity in Response to GDF-5—We next investigated whether type I and type II receptors are capable of...
signaling upon binding GDF-5 using a p3TP-Lux promoter-reporter construct (54). R mutant Mv1Lu cells were transfected with type I and/or type II receptors, together with p3TP-Lux, and stimulated or not stimulated by GDF-5. Since transfection of empty pSV7d vector showed no luciferase response to GDF-5 (Fig. 6, first set of bars), these cells were used for the analysis of transfected GDF-5 receptor signaling activity. Cells transfected with ActR-II or BMPR-II alone did not respond to GDF-5 (Fig. 6). Cells transfected with BMPR-IA or BMPR-IB alone showed a very weak response to GDF-5, which may be ascribed to the presence of endogenous activin and BMP type II receptors in the R mutant cells (26, 36). When p3TP-Lux was co-transfected with ActR-II and BMPR-IB, transcriptional activation was clearly observed after stimulation by GDF-5 (Fig. 6). BMPR-IA also showed a less but significant increase in luciferase activity in the presence of ActR-II. Similarly, co-transfection of BMPR-II and BMPR-IA did not show significant transcriptional activation (Fig. 6). In the R mutant cells co-transfected with ActR-IIIB1 and type I receptors (ActR-I, BMPR-IA, and BMPR-IB), the activation of transcription by GDF-5 was not detected (data not shown).

DISCUSSION

In the present paper, we first tried to find the cell lines that respond to GDF-5 in order to identify the signaling receptors for GDF-5. In contrast to BMP-2, BMP-4, and OP-1/BMP-7, GDF-5 did not efficiently induce the ALP activity in most cell lines, including MC3T3-E1, ROS17/2.8, and C2C12 cells. Only the osteoprogenitor-like cell line, ROB-C26, could respond to GDF-5 (Fig. 1, A and B). These data suggested to us that the bioactivity of GDF-5 can be observed in limited cell types, and the receptor-binding profile of GDF-5 may be different from those of other BMPs. We then investigated the receptors in the ROB-C26 cells using antibodies against known serine/threonine kinase receptors. Interestingly, GDF-5 bound to only BMPR-IB and BMPR-II in this cell line but not to the other serine/threonine kinase receptors, including BMPR-IA (Fig. 2A). Since BMP-2 showed a similar binding profile in the ROB-C26 cells (Fig. 2B), this cell line may predominantly express BMPR-IB and BMPR-II compared with the other receptors.
Furthermore, the binding of $^{125}$I-GDF-5 and $^{125}$I-BMP-2 to BMPR-IB was competed by unlabeled GDF-5 or BMP-2 (Fig. 2C), indicating that the binding site to BMPR-IB is shared by these ligands. MC3T3-E1 cells are known to respond to BMP-4 and OP-1/BMP-7. In this cell type, ActR-I and BMPR-IA were shown to bind $^{125}$I-OP-1/BMP-7 and $^{125}$I-BMP-4, respectively (27). However, $^{125}$I-GDF-5 did not efficiently bind to MC3T3-E1 cells, and we could not detect the cross-linked complexes with $^{125}$I-GDF-5 in this cell line using any of the serine/threonine kinase receptor antisera (data not shown).

We then investigated the binding of GDF-5 to cell lines of nonskeletal origins. In U-1240 MG glioblastoma cells and Mv1Lu mink lung epithelial cells, GDF-5 bound to BMPR-IB among the six type I receptors (Fig. 3). These cells have previously been shown to endogenously express ActR-I and BMPR-IA (27), but the cross-linked complexes with $^{125}$I-GDF-5 were not immunoprecipitated by the antisera against ActR-I or BMPR-IA. Moreover, we found that $^{125}$I-BMP-2 bound to BMPR-IA as well as BMPR-IB in the Mv1Lu cells (data not shown). Taken together, the binding profile of GDF-5...
in nontransfected cells is different from that of BMP-2, BMP-4, or OP-1/BMP-7; i.e. GDF-5 preferentially binds to BMPR-IB but not to ActR-I and BMPR-IA.

We next studied the binding of GDF-5 in COS-1 cells transfected with the receptor cDNAs. Similar to BMP-4 and OP-1/BMP-7, BMPR-IB bound GDF-5 without the transfection of type II receptors. However, other type I receptors did not bind GDF-5 in the absence of type II receptors (Fig. 4). Binding of GDF-5 to the type II receptors was similar to that of OP-1/BMP-7; i.e. GDF-5 bound to BMPR-II as well as ActR-II and ActR-IIb1 (Fig. 4). When the type II receptors were co-transfected with the type II receptors, the binding profiles were complicated; GDF-5 formed complexes only with BMPR-IB and also with BMPR-IA in the presence of ActR-II (Fig. 5B). In the presence of ActR-IIb1, binding of GDF-5 to ActR-I, BMPR-IA, and BMPR-IB could be seen, but BMPR-IB was not likely to form a tight complex with ActR-IIb1, because co-immunoprecipitation of neither ActR-IIb1 by BMPR-IB, respectively, may have broad physiological functions in different tissues.

Compatible with the limited expression of GDF-5, the mutations in the GDF-5 gene result in the abnormalities only in skeletal tissues. Thus, GDF-5 or other highly related proteins may play important roles in nonskeletal tissues.

Among the TGF-β superfamily, BMPs are classified by their ability to form bone and cartilage in vivo. However, BMPs include heterogeneous members, and they can be subdivided into subgroups based on their amino acid sequence similarities. Moreover, the biological activities appear to be different among the members in the BMP family. Present data showed that binding profiles of GDF-5 are more limited than those of BMP-2, BMP-4, and OP-1/BMP-7, which suggests that different biological functions of GDF-5 both in vitro and in vivo. Future studies including the comparison of the receptor binding properties of the other members in the BMP family, will be needed to understand the in vivo functions of various members of the BMP family.

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