Interaction between Soluble Type I Receptor for Bone Morphogenetic Protein and Bone Morphogenetic Protein-4*

(Received for publication, November 22, 1996)

Tohru Natsume‡‡, Shuichiro Tomita‡, Shunichiro Iemura‡, Naoki Kinto***, Akira Yamaguchi, and Naoto Ueno‡‡

From the ‡‡Research and Development Center, Nippon Meat Packers, Inc., 3-3 Midorigahara, Tsukuba, Ibaraki 300-26, Japan, ‡Department of Insect Genetics and Breeding, National Institute of Sericultural and Entomological Science, 1-2 Ohiwashi, Tsukuba, Ibaraki 305, Japan, ‡Department of Oral Pathology, School of Dentistry, Showa University, Shinagawa-ku, Tokyo 142, Japan. **Department of Orthopedic Surgery, School of Medicine, Niigata University, Niigata 951, Japan, and ‡‡Faculty of Pharmaceutical Sciences, Hokkaido University, Sapporo 060, Japan

Bone morphogenetic proteins (BMPs) are multifunctional proteins that comprise the largest subfamily of the transforming growth factor-β. These proteins bind to types I and II serine/threonine kinase receptors. Ligand-induced heteromeric dimerization of these receptors is the key event in initiation of biological responses. We report here large-scale expression and purification of extracellular domain of the type I receptor for BMP-2/4, using a silkworm expression system. This soluble form of BMP receptor (sBMPR) was in monomeric form in solution and bound to BMP-4 but not to activin or transforming growth factor-β1. Surface plasmon resonance studies showed that kinetic parameters of sBMPR for BMP-4 consisted of a relatively rapid association rate constant ($k_a = 3.81 \pm 0.19 \times 10^4 \text{ s}^{-1} \text{ M}^{-1}$) and an extremely slow dissociation rate constant ($k_d = 3.69 \pm 0.26 \times 10^{-4} \text{ s}^{-1}$). From these two kinetic parameters, affinity was determined to be similar to that of the intact membrane-associated receptor expressed on COS cells. sBMPR inhibited the alkaline phosphatase activity in BMP responsive cell lines such as mouse osteoblastic cell MC3T3-E1 and bone marrow stromal cell ST2. These data indicate that the extracellular domain of type I receptor for BMP-2 and BMP-4 is sufficient for high-affinity binding to its ligands and should prove useful in understanding the role of BMP-2/4 in vivo, because a suitable high-affinity anti-BMP antibody has yet to be developed.

Bone morphogenetic proteins (BMPs), originally identified as proteins to induce endochondral bone formation in ectopic extraaskeletal sites in vivo (1), are the largest subfamily of the transforming growth factor-β (TGF-β). Of over a dozen of these BMP members (2, 5), BMP-2 and BMP-4 (vertebrate ortholog of Drosophila decapentaplegic) appear to play important roles in embryogenesis and body patterning (4). We reported that a Xenopus homologue of BMP-2 and BMP-4 present in developing Xenopus embryos (5–8) regulates dorsal-ventral patterning during mesoderm induction (9). In addition to the dorsal-ventral specification, BMP-2 and BMP-4 are also involved in later stages of development, e.g. differentiation of neural cells (10–13), regulation of patterning in the limb bud (14, 15), and epithelial-mesenchymal interactions during organogenesis (16).

Receptors for BMPs are a family of transmembrane serine/threonine kinases (17). These receptors are divided into two distinct classes, type I (18–20) and type II receptors (21, 22). Like receptors for other TGF-β-related proteins, heteromeric dimerization of these receptors, induced by binding to their ligands, is responsible for initiating biological responses (22–25). However, a cross-linking study showed that when transiently expressed, these type I receptors are capable of binding to BMP-2 and BMP-4, without co-expression of the type II receptor (18–20, 26). The type II receptor for BMPs bound to ligands weakly, but the binding was facilitated by the presence of type I receptors for BMPs (21, 22, 24). When the dominant-negative form of type I receptor, lacking the serine/threonine kinase domain, was expressed in ventral blastomeres of Xenopus embryos, the BMP-4 signal was blocked, resulting in formation of a secondary body axis (19, 26). These findings indicate that the extracellular domain (ECD) of type I receptor is sufficient to mediate stable binding to BMPs and subsequent formation of a heteromeric complex with the intact (endogenous) type II receptor. Thus, a dominant-negative experiment would be pertinent to study the biological function of BMP-2 and BMP-4 by specifically disrupting the BMP signal. However, properties of the dominant-negative truncated type I receptor, its binding affinity, specificity, and requirement of other components for binding to ligands remain to be clarified.

In an attempt to address these problems and to determine the physiological role of type I receptors for BMP signaling, we produced a soluble form of the ECD of type I receptor fused to the myc-epitope sequence (sBMPR), using a silkworm expression system. This system, first reported by Maeda et al. (27), facilitates a high level of expression of the active form of recombinant proteins that often become insolubilized in the Escherichia coli expression system as an inclusion bodies form. Using this system, a milligram quantity of the sBMPR was expressed, and the purified sBMPR showed a high affinity for recombinant Xenopus BMP-4 (rxBMP-4). Using a surface plasmon resonance biosensor, sBMPR bound specifically to rxBMP-4 immobilized on the sensor chip surface but not to activin A or the TGF-β1 immobilized surface. Kinetic analysis showed that binding of sBMPR to rxBMP-4 consisted of a rapid association rate constant ($k_a = 3.81 \pm 0.19 \times 10^4 \text{ s}^{-1} \text{ M}^{-1}$) and...
an extremely slow dissociation rate constant ($k_3 = 3.69 \pm 0.26 \times 10^{-4} \text{s}^{-1}$). We also asked if sBMPR would compete with membrane-associated BMP receptors in culture cells and act as an antagonist of BMPs. The sBMPR inhibited ALP activity in BMP-responsive cell lines such as MC3T3-E1 cells and ST2 cells.

**MATERIALS AND METHODS**

**Construction of sBMPR and Xenopus BMP-4 (sBMP-4)—**To express the soluble form of the BMP receptor (sBMPR), a truncated mutant of cDNA that lacks both transmembrane and serine/threonine kinase domains (coding sequences for amino acids 1–152) was amplified from mTFR11 cDNA cloned in BlueScript SK(+) by polymerase chain reaction (PCR), using a universal forward primer and a TFR11myc reverse primer. The TFR11myc primer, 3'-GAACTACCGCTCGATG-GCTCTCCTGTCCTGACTAGGCTCCCTCCTGGACATC, was designed so that the myc epitope sequence (EQKLISEEDL) would be added at the carboxyl terminus of the mutant protein (28). The PCR reaction was performed in a volume of 100 µl containing 10 µl of the reverse transcriptase reaction, 10 mM KCl, 20 mM Tris-HCl, pH 8.0, 10 mM (NH₄)₂SO₄, 6 mM MgSO₄, 0.1% Triton X-100, 1 unit of PuF DNA polymerase (Stratagene), 40 ng of template DNA, and 50 pmol each of the forward and the reverse primers. After 30 cycles of denaturation (95°C, 1 min), annealing (55°C, 45 s), and extension (72°C, 2 min), the PCR product was recovered from low melting temperature agarose gels and desalted on a RPC C2/C18 column (Pharmacia Biotech Inc.). The column was eluted with a linear gradient from 0 to 1.0M sodium chloride. The immunoreactive fraction in 0.5 M NaCl elution was further separated by gel filtration performed on Superdex 200 (Pharmacia). Following these steps, the fraction, containing immunoreactivity of the anti-myc epitope, 9E10 (28) or anti BMP-4 polyclonal antibody Ab383 (29), was loaded on a heparin-Sepharose CL-6B column (Pharmacia), which was eluted by 0.25M sodium chloride containing 4 M urea. The active fraction was loaded on a phenyl-Sepharose column (Pharmacia, Tokyo, Japan). The 9E10 cell line, which was purified from the growth medium of 9E10, using a protein A affinity column.

**Binding experiments**—Surface plasmon resonance studies were performed using the BIAcore2000 (Biacore). The purity of the protein was >98%, determined by silver staining of SDS-PAGE (data not shown).

**Fig. 1. Production and purification of sBMPR in silkworm larvae.** A, the fifth instar larvae of the silkworms were infected with sBMPR-recombinant virus (lanes 4 and 7), and the hemolymph was recovered as described under “Material and Methods.” 0.5 µl of the hemolymph was separated by SDS-PAGE, under reducing conditions (lanes 2–4), and analyzed by Western blots with the monoclonal antibody against the myc-epitope, 9E10 (lanes 5–7). For control experiments, the hemolymph of noninfected (lanes 2 and 5) and wild virus-infected larvae (lanes 3 and 6) was also examined. An arrowhead and arrow indicate bands of the sBMPR produced in hemolymph infected with the recombinant virus. The molecular markers (phosphorylase B, 97,400; bovine serum albumin, 66,200; hen egg ovalbumin, 45,000; bovine carbonic anhydrase, 31,000; soybean trypsin inhibitor, 21,500; and hen egg lysozyme, 14,400, Bio-Rad) are shown in lane 1. Purified sBMPR was analyzed by 15% SDS-PAGE, under reducing (lane 2) and nonreducing conditions (lane 3). The gel was stained with Coomassie Blue. The molecular markers are the same as in lane 1.

**Western Blot Analysis**—Immunodetection of sBMPR and rBMP-4 was performed by Western transfer of the fifth instar larvae of the silkworms (Kita) fed artificial diets (Nohsannkoh, Japan) containing 100 mM KCl, pH 7.4. The immunoreactive sBMPR was determined by measuring the absorbance in a 1-cm-path length cell at 280 nm. Chloride. The active fraction in 0.5 M NaCl elution was pooled, acidified to pH 3.0 with CF₃COOH (TFA), and concentrated on a µRPC C2/C18 column (Pharmacia, SMARTSystem). The column was preequilibrated with 0.1% TFA and was eluted with a linear gradient from 0 to 80% acetonitrile containing 0.1% TFA. The main peak eluted at 60% acetonitrile was pooled and stored at 4°C until use. The purity of the protein was >98%, determined by silver staining of SDS-PAGE (data not shown).

**Peptide Growth Factors and Antibodies**—Purified recombinant human activin produced in insect cells was a gift from Dr. I. Eto (Ajinomoto, Inc., Kawasaki, Japan). Recombinant human TGF-β1 was purchased from King Jyohzo (Tokyo, Japan). The 9E10 cell line, which contains anti-myC antibody 9E10 analyzed by Western blot, was dialyzed against Tris-buffer (100 mM Tris-HCl, pH 7.4) and loaded on a ResourceQ column (Pharmacia) preequilibrated with the same buffer. The column was eluted with a linear gradient from 0 to 1.0 M sodium chloride. The immunoreactive fraction in 0.5 M NaCl elution was further separated by gel filtration performed on Superdex 75 (Pharmacia) equilibrated in Tris-buffered saline (50 mM Tris-HCl, 100 mM KCl, pH 7.4). The immunoreactive sBMPR was eluted as the main peak of low molecular mass (<20 kDa). The protein concentration of purified sBMPR was determined by measuring the absorbance in a 1-cm-path length cell at 280 nm.

**Surface Plasmon Resonance Studies**—Binding experiments and kinetic analysis were performed using the BIAcore2000 (Biacore). The basic principles and its use have been documented (32, 33). Purified rBMP-4 described above, recombinant human activin, and recombinant human TGF-β1 were prepared in immobilization buffer (10 mM acetic acid, pH 4.8) at a concentration of 10 µg/ml and were immobilized on sensor chips (CM5, certified grade, Biacore) at a flow rate of...
20 μl/min at 25 °C for 150 s by the amine coupling method (32). The immobilization levels for rxBMP-4, activin, and TGF-β1 were 1031, 1543, and 1012 RU, respectively. For binding studies, sBMPR was injected over a range of concentrations between 10 nM and 12.5 μM at 25 °C for 60–150 s. Before the analysis, the flow rate of the analyte injection was optimized to minimize mass transport limitations (33). A flow rate of 30 μl/min was found to be sufficient to overcome the mass transport limitation (data not shown). The kinetic parameters, association rate constant (kₐ) and dissociation rate constant (kₐ), were determined from three independent experiments, using BIAevaluation software version 2.1 (Biacore). The buffer for sample dilution and running buffer for BIAcore2000 was HBS (50 mM HEPES, 150 mM NaCl, 3.4 mM EDTA, and 0.005% Nonidet P-40, pH 7.4). Prior to data collection, several methods for surface regeneration after ligand binding were evaluated. Injection of 100 mM HCl (20 μl) efficiently removed the bound proteins and preserved the binding capacity of the sensor chip surface. rxBMP-4 surfaces were stable for over 100 binding and regeneration cycles (data not shown).

**Assessment of Biological Activity of sBMPR**—Biological activity of sBMPR was examined using two different cell lines. MC3T3-E1 cells were provided by Dr. M. Kumegawa. ST2 cells were obtained from the RIKEN Cell Bank (Tsukuba, Japan). These cells were inoculated at a density of 3 x 10³ cells/well in 24-well plates and cultured with α-modified Minimum Essential Medium (Life Technologies, Inc., Grand Island, NY) containing 10% fetal bovine serum (Life Technologies, Inc.) and antibiotics (100 units/ml of penicillin-G and 100 μg of streptomycin). To examine the biological activity of sBMPR, ALP activity of MC3T3-E1 cells was examined. Various concentrations of sBMPR were added to the medium on day 1 of the culture, and ALP activity was determined on day 6 of the culture, using an established technique (34). ST2 cells were grown in the presence of rxBMP-4 (100 ng/ml) and various concentrations of sBMPR. ALP activity was determined on day 3 of culture.
RESULTS AND DISCUSSION

To confirm the synthesis of sBMPR in the silkworm expression system we used, 0.5 ml of silkworm hemolymph was subjected to electrophoresis on a 15% SDS polyacrylamide gel. Under reducing conditions, a diffuse band with a relative molecular mass of 120 kDa was observed for hemolymph recovered from larvae infected with sBMPR-recombinant virus but not in noninfected or in wild virus-infected hemolymph (Fig. 1A, lane 2–4). Western blot analysis showed that this species reacted strongly with the anti-myc monoclonal antibody 9E10 (Fig. 1A, lane 7), thereby suggesting the production of myc-epitope tagged sBMPR in silkworm larvae. To obtain a large quantity of sBMPR, 100 ml of hemolymph were collected from 200 larvae infected with the recombinant virus. The recovered hemolymph was precipitated with 50% ammonium sulfate, and sBMPR was purified using hydrophobic, ion-exchange, and size-exclusion chromatography, as described under “Material and Methods.” Using this expression system, a milligram quantity of sBMPR (∼5 mg) was obtained.

The N-terminal amino acid sequence of purified sBMPR was analyzed using an automated pulse liquid-phase sequencer. A complete single amino acid sequence of 15 amino acid residues was determined to be “GMKSDLQKFPENGY.” This sequence was a perfect match with the coding sequence for amino acids 34–49 deduced from the cDNA sequence. However, the signal peptide of TFR11 had been predicted to be cleaved after S19 (19). It cannot be ruled out that the observed N-terminal sequence is the original cleavage site in vivo; several recombinant proteins expressed in insect cells were found to be processed at different positions of natural proteins (31, 35).

In SDS-PAGE analysis, the purified sBMPR migrated on the gel at a molecular mass of ∼16 and ∼20 kDa, under nonreducing and reducing conditions, respectively (Fig. 1B). Molecular masses of the observed bands in SDS-PAGE were greater than that predicted by cDNA sequence (129 residues; 1,430 Da). This was presumably the result of glycosylation on the two potential N-glycosylation sites of the molecule (19). A molecular mass of 16 kDa was also determined by matrix-assisted laser desorption mass spectrometry, indicating that sBMPR was monomeric (data not shown), despite the presence of 10 conserved cysteine residues of ECD. This was further supported by a global analysis of equilibrium sedimentation, using an analytical ultracentrifuge. Data from this yielded a single molecular mass of 16 ± 0.5 kDa and indicated that sBMPR was a monomer in solution and did not aggregate further.2 Recently, we confirmed that all cysteines of ECD formed five intramolecular disulfide bridges and no unpaired cysteine residues.3 Differences in molecular mass under reducing conditions in SDS-PAGE analysis would be the result of disruption of these intramolecular disulfide bonds.

For binding assay, purified recombinant rxBMP-4 was immobilized on sensor chips of BIAcore, and 5 μg/ml of sBMPR were passed over the sensor chip. During the injection of sBMPR, a rising slope of the RU was evident, and after the injection, the RU decreased slowly. However, this change in sensorgram did not occur when the sBMPR was injected over either an activin or a TGF-b1 immobilized sensor chip surface. This was identical to the control injection with mock-coupled sensor chip surface in the absence of proteins (Fig. 2A). To detect the myc epitope tag of

---

2 T. Natsume, T. Hatta, N. Ueno, and Y. Kobayashi, manuscript in preparation.
3 Y. Nishiuchi, T. Natsume, T. Yamazaki, T. Hatta, N. Ueno, and Y. Kobayashi., manuscript in preparation.

---

FIG. 3. Kinetic analysis of sBMPR binding to BMP-4. A, to determine rate constants of the sBMPR for BMP-4, sBMPR was injected over rxBMP-4 immobilized on the sensor chip at different concentrations (0.1–12.5 μM) at a flow rate of 30 μl/min. Arrowheads, the initiation and termination of the injections. A single exponential binding model (A + B = AB) was fitted to the binding curves shown in A. B and C are representative examples of the curve fitting of the association and dissociation phases, respectively. The actual data points are shown as symbols, and the fitted curves are shown as solid lines.
sBMPR, injection of anti-myc monoclonal antibody 9E10 followed the injection of sBMPR over the xBMP-4 surface. After binding of sBMPR to rxBMP-4 immobilized on the sensor chip, a second increase of 1000 RU was observed by sequential injection of 9E10 (3 μg/ml). When 9E10 was injected alone (blank surface) or injected with a 10-fold molar excess of myc epitope peptide (EQKLI-SEEDL), this second response during the injection of 9E10 was precluded (Fig. 2B), indicating that the second response was specific binding of 9E10 to the protein containing the myc epitope sequence, that is sBMPR. These results clearly suggest that the ECD of TFR11 was sufficient for binding to BMP-4 and did not bind either activin or TGF-β1. The myc epitope sequence at the amino terminus of sBMPR was well recognized by 9E10, forming a stable complex with sBMPR and rxBMP-4 on the sensor chip.

To determine kinetic parameters, association rate constant and dissociation rate constant, we next injected increasing concentrations of sBMPR over immobilized xBMP-4 on the sensor chip (0.1–12.5 μM). Sensorgrams consisted of a rapid increase in RU during injections and an extremely slow decrease of dissociation phase (Fig. 3A); curves were analyzed using nonlinear least squares methods. Dissociation phase (140–200 s) gave a good fit to a single exponential interaction model at all concentrations, and the dissociation rate constant, k_d, was calculated to be 3.69 ± 0.26 × 10^{-2} s^{-1} (Fig. 3C).

However, the association phase (80–130 s) was fitted to a single exponential interaction model only at a low concentration range (<3 μM), and the association rate constant, k_a, was estimated to be 3.81 ± 0.19 × 10^4 s^{-1} M^{-1} (Fig. 3B). The apparent equilibrium dissociation constant (K_d) determined from these two rate constants (k_d/k_a) was 9.6 nM. The affinity determined by this method is similar to K_d values (0.5–3.5 nM) for intact type I receptors for BMPs binding to their ligand (18, 19, 26).

Determination of kinetic parameters of the sBMPR/BMP-4 interaction was also done in reverse orientation, i.e. with sBMPR immobilized on the sensor chip surface and rxBMP-4 in solution. However, the injected BMP-4 was absorbed in the micro flow channel of the instrument and hardly reached flow cells of the sensor chip surface at neutral pH range (data not shown), probably because of the sticky nature of BMP-4 (1). Thus, using a sBMPR immobilized surface was not feasible for reproducible data collection and quantitative analysis.

We then measured ALP activity of MC3T3-E1 cells to determine if the sBMPR could compete with the membrane-bound BMP receptors on cultured cells and could act as an antagonist of BMPs. MC3T3-E1 cells are well characterized as an osteoblastic cell line that differentiates into mature osteoblasts responding to BMPs and shows high ALP activity. The cells produce several families of BMPs and respond in an autocrine manner. Reverse transcription-PCR blot analysis showed that this cell line expresses BMP-2 and BMP-4 of transcripts.4 Fig. 4A shows the dose-dependent inhibition effect of sBMPR on ALP activity in MC3T3-E1 cells on day 6 of culture. Maximum

---

4T. Natsume, T. Hatta, Y. Kobayashi, and N. Ueno, unpublished observation.

---

**Fig. 4. Inhibitory effect of sBMPR on ALP activity in BMP-responsive cell lines.** MC3T3-E1 and ST2 cells were inoculated at a cell density of 3 × 10^4 cells/well in 24-well plates and cultured, as described under "Materials and Methods." A, the indicated concentrations of sBMPR were added to the culture medium of MC3T3-E1 cells on day 1 of the culture, and ALP activity was determined on day 6. B, ST2 cells were grown in the absence (−) and presence (+) of rxBMP-4 (100 ng/ml) with the indicated concentrations of sBMPR. After 3 days of treatment, ALP activity was determined. Data are expressed as the means (bars, S.E.) of three different wells. B, below concentration indicates the addition of buffer used for sample dilution (50 mM Tris-HCl, 150 mM NaCl, pH 7.4) for control culture.
inhibition was observed with a concentration of 3 µg/ml of sBMPR. Added sBMPR was considered to bind to BMPs secreted into the culture medium and to act as a antagonist by competing with wild membrane-bound receptors; the major type I receptor for BMP expressed on the cell was TFR11 (ALK-3), which was cloned using a cDNA library of MC3T3-E1 cells (19).

Next we asked if sBMPR could block the differentiation of bone marrow stromal cells ST2 into osteoblasts induced by BMP-4 (36). They do not exhibit osteoblastic features, such as an increase in ALP activity, in control culture. However, when the cells are cultured in the presence of rxBMP-4 (100 ng/ml), the level of ALP activity increased 6-fold over that of control culture. This effect of BMP-4 on ALP activity was suppressed when sBMPR was added, dose-dependently (Fig. 4B). In the presence of 1 µg/ml of sBMPR, the ALP activity was reduced to basal level, and 50% inhibition was obtained at about 100 ng/ml of sBMPR (molar ratio of added sBMPR to rxBMP-4 was ~2:1).

sBMPR described here has facilitated structure-function analysis of the ECD of the type I receptor for BMP-2 and BMP-4. Our data show that sBMPR retains high-affinity binding and forms a stable complex with BMP-4 because of an extremely slow dissociation rate constant. Because only the active form (mature form) of BMPs is capable of binding to its receptor (17), the sBMPR might be useful to detect active forms of BMP-2 and BMP-4 as well as for use as a BMP antagonist. An anti-BMP antibody that recognizes active BMP and neutralizes the BMP signal has yet to be developed.

Acknowledgments—We thank Mariko Ohara for editing the manuscript. We acknowledge the support of Minoru Hata and Dr. Katsuhiko Mikoshiba.

REFERENCES

1. Reddi, A. H. (1994) Curr. Opin. Genet. Dev. 4, 737–744
2. Massagué, J., Attisano, L., and Wara, J. L. (1994) Trends Cell Biol. 4, 172–178
3. Wozney, J. M. (1992) Mol. Reprod. Dev. 32, 160–167
4. Harland, R. M. (1994) Proc. Natl. Acad. Sci. U. S. A. 91, 10243–10246
5. Nishimatsu, S., Suzuki, A., Shoda, A., Murakami, K., and Ueno, N. (1992) Biochem. Biophys. Res. Commun. 186, 1487–1495
6. Shoda, A., Murakami, K., and Ueno, N. (1993) Growth Factors 8, 165–172
7. Shoda, A., Murakami, K., and Ueno, N. (1994) Biochem. Biophys. Res. Commun. 198, 1267–1274
8. Ueno, N., Shoda, A., Takebayashi, K., Suzuki, A., Nishimatsu, S., Kikuchi, T., Wakima, M., Fujino, M., and Murakami, K. (1992) Growth Factors 7, 233–240
9. Jones, C. M., Lyons, K. M., Lapan, P. M., Wright, C. V. E., and Hogan, B. J. M. (1992) Development (Camb.) 115, 639–647
10. Hawley, S. H., Wunnenberg-Stapleton, K., Hashimoto, C., Laurent, M. N., Watabe, T., Blumberg, B. W., and Cho, K. W. (1995) Genes Dev. 9, 2923–2935
11. Paralkar, V. M., Weeks, B. S., Yu, Y. M., Kleinman, H. K., and Reddi, A. H. (1992) J. Cell Biol. 119, 1721–1728
12. Perides, G., Hui, G., Ruesger, D. C., and Charness, M. E. (1993) J. Biol. Chem. 268, 25197–25205
13. Shah, N. M., Groves, A. K., and Anderson, D. J. (1996) Cell 85, 331–343
14. Franzen, P., ten Dijke, P., Ichijo, H., Yasuhara, H., Schulz, P., Heldin, C.-H., and Miyazono, K. (1993) Cell 75, 681–692
15. Zou, H., and Niswander, L. (1996) Science 272, 738–741
16. Vainio, S., Karavanova, I., Jowett, A., and Thesleff, I. (1993) Cell 75, 45–58
17. Miyazono, K., ten Dijke, P., Yamashita, H., and Heldin, C. H. (1994) Semin. Cell Biol. 5, 389–398
18. Korning, B. B., Cook, J. S., Welsing, D. H., Ting, J., Tiseman, J., Peere, C. P. E., Olson, C. A., Pequenat, A. L., Ventura, F., Grant, R. A., Chen, G.-X., Wrana, J. L., Massague, J., and Rosenbaum, J. S. (1994) Mol. Cell. Biol. 14, 5961–5974
19. Suzuki, A., Thies, R. S., Yamaji, N., Song, J. J., Wozney, J. M., Murakami, K., and Ueno, N. (1994) Proc. Natl. Acad. Sci. U. S. A. 91, 10255–10259
20. ten Dijke, P., Yamashita, H., Sampath, T. K., Reddi, A. H., Estevez, M., Riddle, D. L., Ichijo, H., Heldin, C.-H., and Miyazono, K. (1994) J. Biol. Chem. 269, 16985–16988
21. Nohno, T., Ishikawa, T., Saito, T., Hosokawa, K., Noji, S., Welsing, D. H., and Rosenbaum, J. S. (1995) J. Biol. Chem. 270, 22522–22526
22. Rosenzweig, B. L., Imamura, T., Okadome, T., Cos, G. N., Yamashita, H., ten Dijke, P., Heldin, C. H., and Miyazono, K. (1995) Proc. Natl. Acad. Sci. U. S. A. 92, 7622–7636
23. Letsou, A., Arora, K., Wrana, J. L., Simin, K., Twombly, V., Jamal, J., Staehling-Hampton, K., Hoffmann, F. M., Gelbart, W. M., and Massague, J. (1985) Cell 60, 899–908
24. Liu, F., Ventura, F., Doody, J., and Massague, J. (1995) Mol. Cell. Biol. 15, 3479–3486
25. Ruberte, E., Marty, T., Nellen, D., Affolter, M., and Basler, K. (1995) Cell 80, 889–897
26. Graff, J. M., Thies, R. S., Song, J. J., Celeste, A. J., and Melton, D. A. (1994) Cell 79, 169–179
27. Maeda, S., Kawai, T., Obinata, M., Fujiwara, H., Horiiuchi, T., Saku, Y., Sato, Y., and Purusawa, M. (1985) Nature 315, 592–594
28. Kolodziej, P. A., and Young, R. A. (1991) Methods Enzymol. 194, 508–519
29. Kobayashi, J., Imanishi, S., Inoue, H., Ohayu, Y., Kamaichi, K., Tsuruoka, N., and Tanaka, S. (1992) Cytotechnology 8, 103–108
30. Felgner, P., Gadek, T., Holm, M., Román, K., Chan, H., Wenz, M., Northrop, J., Ringold, G., and Danielsen, M. (1987) Proc. Natl. Acad. Sci. U. S. A. 84, 7413–7417
31. Ishida, I., Tsujimata, M., Kana, T., Shimamura, A., Tsuura, K., Kanada, S., Katayama, T., Oikawa, S., Matsu, M., Nakashima, T., Kobayashi, J., and Nakazato, H. (1994) J. Biochem. (Tokyo) 115, 279–285
32. Johnson, B., and Lootis, S. (1991) Anal. Biochem. 195, 268–277
33. Karlsson, R., Roos, H., Fagerström, L., and Persson, B. (1994) Methods (Orlando) 6, 99–110
34. Suzuki, A., Nishimatsu, S., Shoda, A., Takebayashi, K., Murakami, K., and Ueno, N. (1993) Biochem. J. 291, 413–417
35. Ueda, Y., Sakurai, T., and Yanai, A. (1993) J. Vet. Med. Sci. 55, 251–258
36. Yamaguchi, A., Ishii, Y., Kintou, N., Yasuhiro, W., Katagiri, T., Wozney, J. M., Rosen, V., and Yoshiki, S. (1996) Biochem. Biophys. Res. Commun. 220, 366–371