Osteogenic Protein-1 Binds to Activin Type II Receptors and Induces Certain Activin-like Effects

Hidetoshi Yamashita,* Peter ten Dijke,** Danny Huylebroeck,‡ T. Kuber Sampath,¶ Maria Andries,‖ James C. Smith,¶ Carl-Henrik Heldin,* and Kohei Miyazono*

*Ludwig Institute for Cancer Research, S-751 24 Uppsala, Sweden; *Laboratory of Molecular Biology (CELEGEN), University of Leuven and Flanders Interuniversity Institute for Biotechnology, and †Laboratory of Cell Pharmacology, University of Leuven, B-3000 Leuven, Belgium; ‡Creative BioMolecules, Inc., Hopkinton, Massachusetts 01748; and ‖Laboratory of Developmental Biology, National Institute for Medical Research, The Ridgeway, London NW7 1AA, United Kingdom

Abstract. Proteins in the TGF-β superfamily transduce their effects through binding to type I and type II serine/threonine kinase receptors. Osteogenic protein-1 (OP-1, also known as bone morphogenetic protein-7 or BMP-7), a member of the TGF-β superfamily which belongs to the BMP subfamily, was found to bind activin receptor type I (ActR-I), and BMP receptors type IA (BMPR-IA) and type IB (BMPR-IB) in the presence of activin receptors type II (ActR-II) and type IIB (ActR-IIB). The binding affinity of OP-1 to ActR-II was two- to threefold lower than that of activin A. A transcriptional activation signal was transduced after binding of OP-1 to the complex of ActR-I and ActR-II, or that of BMPR-IB and ActR-II. These results indicate that ActR-II can act as a functional type II receptor for OP-1, as well as for activins. Some of the known biological effects of activin were observed for OP-1, including growth inhibition and erythroid differentiation induction. Compared to activin, OP-1 was shown to be a poor inducer of mesoderm in Xenopus embryos. Moreover, follistatin, an inhibitor of activins, was found to inhibit the effects of OP-1, if added at a 10-fold excess. However, certain effects of activin, like induction of follicle stimulating hormone secretion in rat pituitary cells were not observed for OP-1. OP-1 has overlapping binding specificities with activins, and shares certain but not all of the functional effects of activins. Thus, OP-1 may have broader effects in vivo than hitherto recognized.

Bone morphogenetic proteins (BMPs)1 were originally identified as proteins that induce bone and cartilage formation in ectopic extraskeletal sites in vivo (reviewed in Reddi, 1992; 1994; Wozney, 1989). In vitro studies have revealed that BMPs have multiple effects on many different cell types, e.g., stimulation of proteoglycan synthesis in chondrocytes (Vukicevic et al., 1989), synthesis of collagen and alkaline phosphatase in osteoblasts (Vukicevic et al., 1989), and differentiation of neural cells (Paralkar et al., 1992; Perides et al., 1994). BMPs also play important roles in the embryonal development, e.g., in ventral mesoderm induction (Dale et al., 1992; Jones et al., 1992). Many proteins belong to the BMP family, including BMP-2 to -6, osteogenic protein-1 (OP-1, also termed BMP-7), OP-2 (BMP-8), and growth/differentiation factor-5 to -7 (Burt and Law, 1994; Kingsley, 1994; Massagué et al., 1994). Some of the members are more closely related to each other than to the other members in the BMP family, and thus, BMPs can be divided into subgroups. BMP-4 and OP-1 belong to different subgroups.

Activins were originally identified as factors in ovarian fluid that stimulate the secretion of follicle stimulating hormone (FSH) from pituitary cells (reviewed in Vale et al., 1990); in contrast, inhibins inhibit FSH secretion. Inhibins are composed of heterodimers of one α chain and one β chain (βA or βB chains). Activins are dimers of β chains; a homodimer of βA chains is denoted activin A. Activins are multifunctional proteins; they stimulate mesoderm induction in Xenopus embryos (Smith et al., 1990; van den Eijnden-Van Raaij et al., 1990; Asashima et al., 1990; Thomsen et al., 1990), stimulate the differentiation of erythroid progenitor cells (Murata et al., 1988; Huylebroeck et al., 1990), and modulate the bone formation by BMP (Ogawa et al., 1992).

1. Abbreviations used in this paper: ActR, activin receptor; ALK, activin receptor-like kinase; BMP, bone morphogenetic protein; BMPR, BMP receptor; BS, bis(sulfosuccinimidyl) suberate; DSS, disucinimidyl suberate; FSH, follicle stimulating hormone; OP, osteogenic protein; PAI, plasminogen activator inhibitor-1; RIA, radioimmunoassay; TBR, TGF-β receptor.
BMPs and activins belong to a larger superfamily, termed the TGF-β superfamily, which contains TGF-β, Müllerian inhibiting substance, and glial cell line-derived neurotrophic factor (reviewed in Burt and Law, 1994; Kingsley, 1994; Massagué et al., 1994). Significant amino acid sequence similarities were observed between the members in the TGF-β superfamily. Moreover, seven cysteine residues are conserved in most of the members in the TGF-β superfamily, suggesting that they have similar three-dimensional structures.

The members of the TGF-β superfamily exert their effects through binding to two types of specific receptors, termed type I (molecular mass of ~53 kD) and type II (~70 kD) receptors (Massagué et al., 1994; Mathews, 1994; ten Dijke et al., 1994a). Types I and II receptors form heteromeric receptor complexes after ligand binding and are indispensable for signal transduction (Wrana et al., 1992, 1994; Inagaki et al., 1993; Attisano et al., 1993; Frančen et al., 1993). Two different forms of activin type II receptors, ActR-II and ActR-IIB, have been cloned and shown to have intracellular serine/threonine kinase domains (Mathews and Vale, 1991; Attisano et al., 1992; Mathews et al., 1992). The TGF-β type II receptor (TβR-II) is also a transmembrane serine/threonine kinase (Lin et al., 1992). DAF-4 from Caenorhabditis elegans serves as a type II receptor for BMP-2, BMP-4, and OP-1, although signaling activity after binding of BMPs has not been demonstrated (Estevez et al., 1995; ten Dijke et al., 1994b).

A series of serine/threonine kinase receptors have been cloned and denoted activin receptor-like kinase (ALK)-1 through ALK-6 by us (Frančen et al., 1993; ten Dijke et al., 1993, 1994c). ALKs have been shown to serve as type I receptors for members of TGF-β superfamily (Table I). ALK-5 is a TGF-β type I receptor (TβR-I) (Frančen et al., 1993; Bassing et al., 1994; ten Dijke et al., 1994c), and ALK-2 and ALK-4 are activin type I receptors (ActR-I and ActR-IB, respectively) (Ebner et al., 1993a; Attisano et al., 1993; Tsuchida et al., 1993; ten Dijke et al., 1994c; Cárcamo et al., 1994). In addition, ALK-3 and -6 have recently been shown to be type I receptors for the proteins in the BMP family (BMPR-IA and BMPR-IB, respectively) (ten Dijke et al., 1994b; Koenig et al., 1994; Graff et al., 1994; Suzuki et al., 1994). BMPR-IA and -IB bind OP-1 and BMP-4 in the presence of DAF-4, although the binding of OP-1 to BMPR-IA is weaker than that of BMP-4. Furthermore, ActR-I can bind OP-1 but not BMP-4 in the presence of DAF-4 (ten Dijke et al., 1994b).

A certain redundancy in the ligand binding of the type I receptors has been reported (Ebner et al., 1993a; Attisano et al., 1993; ten Dijke et al., 1994c). In contrast, ligand binding to the type II receptors has been shown to be highly specific, i.e., TβR-II binds only TGF-β, ActR-II, and ActR-IIB bind only activin and inhibit, and DAF-4 binds only BMPs (Mathews and Vale 1991; Attisano et al., 1992; Mathews et al., 1992; Lin et al., 1992; Estevez et al., 1993) (Table I). However, since ActR-I acts as a type I receptor for activins in the presence of ActR-II or ActR-IIB (Attisano et al., 1993; Ebner et al., 1993a; Tsuchida et al., 1993; ten Dijke et al., 1994c), and also binds OP-1 in the presence of DAF-4 (ten Dijke et al., 1994b), we investigated in the present work whether OP-1 can bind to ActR-IIs and transduce activin-like signals.

**Materials and Methods**

**Cell Culture**

Mink lung epithelial cells (MvLu) and COS-1 cells were obtained from American Type Culture Collection (Rockville, MD). Chemically mutagenized MvLu cell line (R mutant, clone 4-2) (Laiho et al., 1990) was a gift from M. Laiho (University of Helsinki, Helsinki, Finland) and J. Massagué (Memorial Sloan-Kettering Cancer Center, New York). The cells were cultured in DME containing 10% fetal bovine serum and antibiotics (100 U/ml penicillin and 50 μg/ml streptomycin) in 5% CO2 atmosphere at 37°C. A human erythroleukemia cell line, K562 (American Type Culture Collection), was cultured in RPMI-1640 buffered with 25 mM Hepes buffer containing 10% fetal bovine serum and antibiotics. Rat pituitary cells were obtained from 14-d-old female Wistar rats. The rats were decapitated and pituitaries were cut into small tissue blocks and enzymatically dispersed as described previously (Deneq et al., 1989). For FSH-release experiments, cells were seeded in 24-well tissue plates at a density of 2 × 104 cells/100 μl/well. After an adhesion period of about 30 min, 1 ml of serum-free culture medium was added per well. The serum-free medium consisted of DME and Ham’s F-12 (1:1 of vol/vol), 15 mM Hepes buffer, 20 μM ethanolamine and 20 mM sodium selenite (special powder mixture prepared by Gibco Europe, Paisley, UK) to which were added 5 mg/ml bovine serum albumin prepared from a 30% stock solution (Gibco), 1 mg/ml NaHCO3, 8 μg/ml transferrin (Gibco), 5 μg/ml insulin (Boehringer Mannheim Biochemicals, Indianapolis, IN), 1 μg/ml catalase (Serva, Heidelberg, Germany), 10 μM ethanol, 35 μg/ml penicillin, 50 μg/ml benzylpenicillin, 50 μg/ml aminoglycoside.

| Table I. Mammalian Serine/Threonine Kinase Receptors for the TGF-β Superfamily |
|-----------------|-----------------|-----------------|
| **Designation** | **Ligands** | **Other designations** |
| **Type I receptors** | | |
| ActR-I | activins, OP-1* | ALK-2 (ten Dijke et al., 1993), SKR-1 (Matsuzaki et al., 1993), Tsk-7L (Ebner et al., 1993b), R1 (He et al., 1993) |
| ActR-IB | activins, OP-1* | ALK-4 (ten Dijke et al., 1993), SKR-2 (Xu et al., 1994), R2 (He et al., 1993) |
| TβR-1 | TGF-β | ALK-5 (Frančen et al., 1993), B4 (He et al., 1993) |
| ALK-1 | unknown | TSR-1 (Attisano et al., 1993), R3 (He et al., 1993) |
| BMPR-1A | OP-1, BMP-4, BMP-2 | ALK-3 (ten Dijke et al., 1993), BRK-1 (Koenig et al., 1994) |
| BMPR-1B | OP-1, BMP-4 | ALK-6 (ten Dijke et al., 1994c) |

*Binding has been shown in the present report.

1Redundancy in the binding of TGF-β and activin to type I receptors have been observed; TGF-β and activin are listed in this table only if they are known to transduce signals (Attisano et al., 1993; Frančen et al., 1993; Bassing et al., 1994; ten Dijke et al., 1994c; Cárcamo et al., 1994).

2The binding of OP-1 to BMPR-1A is weaker than that of BMP-4 (ten Dijke et al., 1994c).
streptomycin, 0.8 μg/ml phenol red (Gibco), 4 mM dexamethasone, and 0.05 mM triiodothyronine. For cross-linking experiments, cells were seeded in six-well plates in a density of 3.33 × 10⁶ cells/ml/well. They were allowed to adhere for about 30 min, and then 2 ml of DME supplemented with 10% fetal bovine serum, 1 mM NaHCO₃ and antibiotics was added to each well. Pituitary cells were cultured in a humidified CO₂ (1.5%) air incubator at 37°C.

Recombinant Proteins

Recombinant human BMP-4, recombinant human TGF-β1 and recombinant human activin A were obtained from A. H. Reddi (Johns Hopkins University, Baltimore, MD), H. Ohashi (Kirin Brewery Company, Maebashi, Japan) and Y. Eto (Ajinomoto Company, Inc., Kawasaki, Japan), respectively. Recombinant bovine activin A used in mesoderm inducing assays was obtained from Innovogenics. Recombinant human follistatin (B4384) was obtained from the National Hormone and Pituitary Program (Rockville, MD).

Recombinant human OP-1 was obtained as described (Ozkaynak et al., 1990; Sampath et al., 1992). In order to confirm that recombinant human OP-1 does not contain activin-like molecules, OP-1 was radiolabeled with 125I (see below) and analyzed by SDS-gel electrophoresis. 125I-OP-1 was observed as multiple components with molecular masses of 30–38 kDa under nonreducing condition and 16–19 kDa under reducing condition. In contrast, 125I-activin A was observed as a 25-kDa component under nonreducing condition and a 13-kDa component under reducing condition. Analysis of OP-1 by reverse phase high performance liquid chromatography revealed three major components eluting very closely to each other. Amino acid sequencing of these components revealed sequences only of mature OP-1; no activin sequences were found.

Recombinant human soluble OP-1, which is a complex containing the NH₂-terminal pro-domain and the mature OP-1, was obtained as described (Jones et al., 1994).

Transient Transfection of cDNAs

cDNAs for type I receptors were cloned as described (Franzén et al., 1993; ten Dijke et al., 1993, 1994c). ActR-II cDNA was a gift from L. S. Mathews and W. W. Vale (Salk Institute, San Diego, CA). ActR-IIB, cDNA and pVP-Lux promoter-reporter construct were obtained from L. Massague, J. L. Wrana and L. Attisano (Memorial Sloan-Kettering Cancer Center). For transient transfection, cDNAs for type I or type II receptors subcloned into pSv7T (Truet et al., 1985), pCDNA I (Invitrogen, San Diego, CA) or pCMV5 (Anderson et al., 1989) expression vectors were used. These plasmids and p3TP-Lux promoter-reporter construct (3 μg for each) were transfected into COS-1 or R mutant MvLu cells by a calcium phosphate precipitation method with a mammalian transfection kit (Stratagene Corp., La Jolla, CA), following the manufacturer’s protocol. In brief, cells were seeded into 6-well cell culture plates at a density of 5 × 10⁵ cells/well and transfected with 5 μg of plasmids on the following day. After overnight incubation, cells were washed four times with phosphate-buffered saline (pH 7.4) and then incubated in DME containing 10% fetal bovine serum and antibiotics. One or two days after, the cells were used for cross-linking and immunoprecipitation studies or transcriptional response assay.

Preparation of Polyclonal Antibodies

Antisera against type I receptors were made against synthetic peptides corresponding to the intracellular juxtamembrane parts of type I receptors as previously reported (Franzén et al., 1993; ten Dijke et al., 1994c). Antiserum against ActR-II (ARC-2), which detects only ActR-II, was generated against a peptide corresponding to the COOH-terminal tail of ActR-II (Ichijo et al., 1993). Antiserum against the intracellular part of ActR-II, which cross-reacts with ActR-IB, was a gift from K. Verschueren (University of Leuven, Leuven, Belgium).

Radiolabeling of Ligands, Binding, Affinity Cross-linking, and Immunoprecipitation

Activin A was iodinated using the chloramine-T method according to Frolik et al. (1984). OP-1 and BMP-2 were iodinated by the same method, but chloramine-T was added two times. Cells were incubated on ice for 2–3 h with 0.2–0.5 nM of 125I-labeled ligands in the presence or absence of unlabeled ligands in the binding buffer (phosphate-buffered saline containing 0.9 mM CaCl₂, 0.49 mM MgCl₂ and 1 mg/ml bovine serum albumin). After incubation, the cells were washed with the binding buffer without bovine serum albumin and cross-linking was done in the same buffer containing 0.28 mM of diisocyanimidyl suberate (DSS) or 1 mM bis(sulfosuccinimidyl) suberate (BS³) (Pierce Chemical Co., Rockford, IL) for 15 min on ice. The cells were washed once with 10 mM Tris-HCl, pH 7.4, 1 mM EDTA, 10% glycerol and 0.3 mM phenylmethylsulphonyl fluoride. The cells were scraped in the same buffer, centrifuged and resuspended in solubilization buffer (150 mM NaCl, 20 mM Tris-HCl, pH 7.4, 1 mM EDTA, 0.3 mM phenylmethylsulphonyl fluoride, 1% Triton X-100, 1% deoxycholate, and 0.2% SDS), followed by incubation for 20 min on ice. Cross-linked materials were then incubated with antiactivin A at 1 h at 4°C. Thereafter, 50 μl of a protein A-Sepharose (Immunosorb A; EC Diagnostics AB Uppsala, Sweden) slurry (50% packed beads in solubilization buffer) was added to immune complexes and the mixture was incubated for 1 h at 4°C. The beads were spun down and washed twice with 20 mM Tris-HCl, pH 7.4, 500 mM NaCl, 1% Triton X-100, 1% deoxycholate, and 0.2% SDS, followed by one time wash in distilled water. The immune complexes were eluted by boiling 3 min in the SDS sample buffer with 10 mM dithiothreitol, and subjected to SDS–gel electrophoresis, followed by autoradiography using Hyperfilm (Amersham Corp., Arlington Heights, IL) or analysis by a PhosphorImager (Molecular Dynamics, Eugene, OR).

[^H]Thymidine Incorporation Assay

MvLu cells were seeded in 24-well plates at a density of 10⁶ cells per well in DME with 5% fetal bovine serum and antibiotics (100 U/ml penicillin and 50 μg/ml streptomycin). After 24 h, the medium was changed into DME with 1% fetal bovine serum and antibiotics containing various concentrations of TGF-β1, OP-1 or activin A in the presence or absence of follistatin. After 16–18 h of incubation, 0.3 μCi of [³H]thymidine (85 Ci/μmol; Amersham Corp.) was added and the cells were incubated for an additional 2 h. Thereafter, the cells were fixed in 5% ice-cold trichloroacetic acid for more than 1 h, and solubilized with 1 M NaOH for more than 20 min. The cell extract was neutralized with 1.5 M HCl and [³H]-radioactivity was determined in a liquid scintillation counter using Ecoscint A (Nacional Diagnostics, Atlanta, GA).

Plasminogen Activator Inhibitor-1 (PAI-1) Assay

PAI-1 induction by TGF-β1, OP-1, and activin A was investigated in MvLu cells and R mutant MvLu cells according to the method reported previously (Laiho et al., 1991; Franzén et al., 1993). In brief, cells were seeded in 6-well cell culture plates and incubated overnight. The cells were exposed to TGF-β1, OP-1, or activin A in serum-free MCDB 104 medium without methionine for 2 h. Thereafter, cultures were labeled with [³⁵S]methionine (40 μCi/ml) for 2 h. The cells were removed by washing on ice once in phosphate-buffered saline, three times in 10 mM Tris-HCl, pH 8.0, 0.5% sodium deoxycholate, and 1 mM phenylmethylsulphonyl fluoride. The cells were washed with phosphate-buffered saline on the following day. Extracellular-matrix proteins were scraped off and extracted into SDS sample buffer containing dithiothreitol and analyzed by SDS–gel electrophoresis, followed by fluorography using Amplify (Amersham Corp.). PAI-1 was identified as a 45-kD protein (Laiho et al., 1991).

Transcriptional Response Assay

R mutant MvLu cells were co-transfected with p3TP-Lux promoter-reporter construct (Wrana et al., 1992; Attisano et al., 1993) with plasmids containing type I or type II receptor cDNAs as described above. Cells were washed with phosphate-buffered saline on the following day. The cells were starved in DME containing 0.1% of fetal bovine serum and antibiotics (100 U/ml penicillin and 50 μg/ml streptomycin) for 6 h and then exposed to TGF-β1, OP-1, or activin A for 24 h. Luciferase activity in the cell lysate was measured using the lucerase assay system (Promega Bio- tec, Madison, WI) according to the manufacturer’s protocol, using a lumino- meter (model 1250; LKB Instruments, Inc., Bromma, Sweden).

Erythroid Differentiation of K562 Cells

Erythroid differentiation was tested using K562 cells. Activin A and OP-1 were subjected to twofold serial dilution in RPMI-1640 medium buffered with 25 mM Hepes buffer and supplemented with 10% fetal bovine serum. Dilution was performed in 96-well plates with final volumes of 100 μl. 1–2 × 10⁵ cells in 100 μl were added to each well of 96-well plate, and cells were incubated for 4–7 d at 37°C. Thereafter, the cells were stained by
FSH Release from Rat Pituitary Cells

The rat pituitary cells were prepared as described above; on the second day the serum-free medium was removed and 1 ml of fresh serum-free medium with test substances was added. After incubation for an additional two days, the medium was collected and FSH concentration was determined by radioimmunoassay (RIA). Each test substance was added to four wells and RIA was performed in duplicate using the FSH-RIA kit (National Institute of Diabetes and Digestive and Kidney Diseases, Rockville, MD) according to the methods of Denef et al. (1989).

Results

Binding of OP-1 to ActR-IIs and Type I Receptors Expressed in COS Cells

To investigate whether ActR-II can serve as a type II receptor for OP-1, cDNAs for ActR-II, and different type I receptors were co-transfected into COS-1 cells. The cells were then incubated with \(^{125}\text{I}-\text{OP-1} \), followed by cross-linking with DSS. Since the cross-linked complexes were difficult to analyze directly by SDS-gel electrophoresis because of high background, cross-linked complexes were immunoprecipitated using antisera against ActR-II or type I receptors. When only ActR-II cDNA was transfected, \(^{125}\text{I}-\text{OP-1} \) bound to ActR-II, as determined by the immunoprecipitation of a cross-linked complex of the expected size using the ActR-II antiserum (Fig. 1 A). ActR-I, BMPR-IA, and BMPR-IB bound \(^{125}\text{I}-\text{OP-1} \) in the presence of ActR-II, but the other type I receptors did not. Co-immunoprecipitation of ActR-II with ActR-I, BMPR-IA, or BMPR-IB could be observed in the presence of \(^{125}\text{I}-\text{OP-1} \) using antisera against ActR-II or type I receptors (Fig. 1 A). In most experiments, binding of \(^{125}\text{I}-\text{OP-1} \) to BMPR-IA was weaker than that to ActR-I or BMPR-IB. Binding of \(^{125}\text{I}-\text{OP-1} \) to ActR-II, one of the spliced forms of the second activin type II receptor (Attisano et al., 1992), was also investigated. ActR-IIb alone bound \(^{125}\text{I}-\text{OP-1} \) weakly, but it bound efficiently in the presence of ActR-I, BMPR-IA, or BMPR-IB (Fig. 1 B). ActR-IIb complexes were co-immunoprecipitated with antisera against type I receptors and vice versa.

For comparison, the binding of \(^{125}\text{I}-\text{BMP-4} \) to ActR-IIs...
was also investigated in COS-1 cells transfected with ActR-IIIs, using the antisera to ActR-II and ActR-IIIB. 

125I-BMP-4 did not bind to ActR-II or ActR-IIIB, efficiently, consistent with the previous report (Attisano et al., 1992).

**Binding of OP-1 to ActR-II on MvILu Cells**

When type I receptors were overexpressed in COS-1 cells together with TβR-II or ActR-II, almost all type I receptors formed complexes with TGF-β or activin, respectively (Ebner et al., 1993a; Attisano et al., 1993; ten Dijke et al., 1994c). These results indicate that ligand binding to receptors expressed at very large numbers in COS-1 cells may not indicate a physiologically significant interaction. Therefore, we have tested the binding of OP-1 to nontransfected, responsive cells with normal receptor numbers. Wild type MvILu cells express ActR-II, ActR-I, ActR-IB (ten Dijke et al., 1994c), and respond to activin A with regard to growth inhibition and PAI-1 production (see below). When the MvILu cells were affinity labeled using 

125I-OP-1 and cross-linked, a 90-kD ActR-II complex could be immunoprecipitated with the antiserum specific for ActR-II (Fig. 2 A). The binding of 125I-OP-1 to ActR-II was decreased to 30% in the presence of more than 50-fold excess of unlabeled OP-1 or activin A. Similarly, the binding of 125I-activin A to ActR-II in MvILu cells was competed with unlabeled activin A, and also with OP-1, although less efficiently (Fig. 2 A).

To compare the binding affinity of OP-1 and activin A to ActR-II, the binding of 125I-labeled ligands was competed with different concentrations of unlabeled ligands. The amounts of radioactivity in the cross-linked complexes containing ActR-II were quantitated using a PhosphorImager. Half maximal competition of 125I-OP-1 (0.5 nM) binding to ActR-II in MvILu cells occurred at about 1.8 nM unlabeled activin A and 5 nM unlabeled OP-1 (Fig. 2 B). Half maximal competition of 125I-activin A (0.2 nM) binding to ActR-II in these cells also occurred at about 2 nM activin and 5 nM OP-1 (Fig. 2 C). Taken together, activin A appears to have two- to threefold higher affinity than OP-1 for binding to ActR-II on MvILu cells.

**Growth Inhibitory Activity of OP-1; Neutralization by Follistatin**

Growth inhibitory activity of OP-1 was compared with those of TGF-β1 and activin A. [3H]Thymidine incorporation into MvILu cells was found to be inhibited by OP-1, but both activin A and OP-1 were about 100-fold less potent than TGF-β1 (Fig. 3 A). Follistatin is a protein that specifically binds activins and neutralizes their biological activities (Ueno et al., 1987; Nakamura et al., 1990). Therefore, as expected, the growth inhibition by activin A was abolished by the addition of equal amount of follistatin. The effect of OP-1 was also neutralized by follistatin; however, the neutralization was observed when follistatin was added at concentrations of more than 10 times of that of OP-1 (Fig. 3 B). Follistatin did not neutralize the growth inhibitory activity of TGF-β1 even when added at a 3,000-fold excess.

**Signal Transduction of OP-1 through ActR-II**

TGF-β1 stimulates the production of PAI-1 protein in

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Yamashita et al. OP-1 Binding to the Activin Type II Receptors
Erythroid Differentiation by Activin A and OP-1

Activin A has been shown to induce differentiation of erythroid progenitor cells, K562 (Huylenbroeck et al., 1990). The effects of OP-1 on K562 cells were studied. Activin A significantly induced differentiation of K562 cells at concentrations over 0.26 nM, whereas OP-1 induced the differentiation at concentrations over 6.7 nM (Fig. 6 A). Both activin A and OP-1 also induced hemoglobin synthesis; in this case activin A was more potent than OP-1 (Fig. 6 B).

In order to identify the receptors which mediate the erythroid differentiation of these cells, binding studies using $^{125}$I-labeled ligands were performed. $^{125}$I-activin A bound to type I and type II receptors for activins, and immunoprecipitation of the cross-linked receptor–ligand complexes by antisera revealed that ActR-I, ActR-IB, and ActR-II were expressed on K562 cells (Fig. 6 C). $^{125}$I-OP-1 bound to ActR-I and ActR-II, but not to ActR-IB or other type I receptors. These results suggest that erythroid differentiation by activin A and OP-1 of K562 cells is, at least in part, induced through ActR-II and ActR-I. Since the erythroid differentiation in K562 cells was more significant by activin A than by OP-1, ActR-IB may also be involved in the stimulation pathway.

FSH-release from Pituitary Cells by Activin A and OP-1

Activin A is known to stimulate the secretion of FSH from pituitary cells (Vale et al., 1990). In agreement with previous findings, a dose-dependent increase of FSH release from rat pituitary cells was observed after the addition of activin A (Fig. 7 A). In contrast, OP-1 did not stimulate the secretion of FSH even at a concentration of 6.7 nM. Moreover, OP-1 did not antagonize the FSH release activity of activin A (Fig. 7 B).

To characterize the receptors involved, the binding of $^{125}$I-labeled ligands to rat pituitary cells was investigated. $^{125}$I-activin A bound rat pituitary cells and formed type II and type I receptor complexes. The complexes between...
Figure 6. Erythroid differentiation of K562 cells by activin A and OP-1 and identification of their receptors. (A) K562 cells were seeded into 96-well plates and exposed to various concentrations of activin A (△) or OP-1 (●) for 4-7 d at 37°C before staining by benzidine. The proportion of hemoglobin positive cells was calculated. (B) Hemoglobin synthesis in K562 cells induced by activin A (△) or OP-1 (●) was examined by the optical measurement of hemoglobin content. K562 cells were exposed to activin A or OP-1 for five days. Hemoglobin content was measured by the optical absorption at 414 nm and expressed relative to that of an unstimulated control. (C) Binding of activin and OP-1 to the type I and type II receptors on K562 cells. K562 cells were affinity labeled using 125I-activin A (Act A) or 125I-OP-1, followed by cross-linking by DSS. The cross-linked complexes were immunoprecipitated with the ActR-II antiserum (ARC-2), or with antisera against type I receptors. Samples were subjected to SDS-gel electrophoresis, followed by autoradiography.

Figure 7. FSH release from pituitary cells by activin A and OP-1 and identification of activin receptors. (A) Rat pituitary cells were seeded in 24-well plates and the medium was changed into fresh medium containing activin A (△) or OP-1 (●). The cells were incubated for another two days and FSH released into the medium was measured by RIA. The amount is expressed relative to an unstimulated control. (B) The possibility that OP-1 antagonizes the FSH release by activin A was investigated. Rat pituitary cells were exposed to different concentrations of OP-1 in the presence (△) or absence (●) of activin A (0.4 nM), and FSH released into the medium was determined as described above. (C) Rat pituitary cells were affinity labeled using 125I-activin A (Act A) or 125I-OP-1, followed by cross-linking by BS3. The cross-linked complexes were immunoprecipitated with the ActR-II antiserum (ARC-2) or with antisera against type I receptors. Samples were subjected to SDS-gel electrophoresis, followed by analysis using a PhosphorImager.

125I-activin A and type II and type I receptors were immunoprecipitated by antisera against ActR-II and ActR-IB, respectively (Fig. 7 C). However, the antisera against ActR-I or the other known type I receptors did not immunoprecipitate the type I receptor complex. 125I-OP-1 also bound to the rat pituitary cells, but very weakly, and a small amount of OP-1--type II receptor complex was immunoprecipitated by the ActR-II antiserum. None of the type I receptor antisera immunoprecipitated the type I receptor complex (Fig. 7 C). Taken together, FSH release from pituitary cells by activin A may be mediated, at least in part, by ActR-II and ActR-IB, which does not transduce signals by OP-1.

Mesoderm-Inducing Activity of OP-1 in Xenopus Embryos

The mesoderm-inducing activity of OP-1 was examined by incubating Xenopus animal caps in different concentrations of OP-1 and comparing the effects of this factor with those of activin A. Mature OP-1 did not induce the mesoderm formation in Xenopus embryos. Therefore, we used the soluble OP-1, which is a complex containing the mature and pro-domains of OP-1, and much more soluble in physiological buffers than the mature OP-1 (Jones et al., 1994). Whereas activin A induced strong elongation of animal caps, the earliest sign of mesoderm induction (Symes and Smith, 1987; Howard and Smith, 1993), soluble OP-1 caused caps only to become slightly misshapen (Fig. 8, A–E). Soluble OP-1 did not appear to inhibit the ability of activin to bring about elongation (Fig. 8 F). Similarly, while activin A caused significant expression of the mesoderm-specific gene Brachyury at concentrations as low as 20 pM, only weak expression of this gene was observed in response to 430 pM soluble OP-1 (Fig. 8 G), and even 4.3 nM had little effect (not shown). Soluble OP-1 at 430 pM did not inhibit the ability of activin A at 2,000 pM to in-
Figure 8. Comparison of the mesoderm-inducing activities of activin A and OP-1. (A–F) Morphological analysis. Animal caps were dissected from *Xenopus* embryos and cultured in the absence of factors (A) or in the presence of 50 U/ml crude human activin A (~400 pM activin) (B) or in the presence of 43 pM (C), 430 pM (D) or 4.3 nM (E) soluble OP-1. Caps in (F) were cultured in the presence of 50 U/ml activin A plus 430 pM soluble OP-1. Explants were photographed at the equivalent of stage 20. (G) Analysis of *Brachyury* (*Xbra*) expression. Animal caps were cultured in the indicated concentrations (in ng/ml) of activin A or soluble OP-1, or in both factors, and frozen for analysis at the equivalent of stage 11. Soluble OP-1 at concentrations of 0.5, 5, and 50 ng/ml corresponds to 4.3, 43, and 430 pM, respectively, and 0.5, 5, and 50 ng/ml activin A is 20, 200, and 2,000 pM, respectively.

Discussion

Type II serine/threonine kinase receptors bind ligands in the absence of type I receptors, whereas type I receptors require type II receptors for ligand binding (Wrana et al., 1992; Inagaki et al., 1993; Ebner et al., 1993b; Attisano et al., 1993; Franzén et al., 1993). Moreover, a certain redundancy has been observed in the binding to type I receptors (Ebner et al., 1993b; Attisano et al., 1993; ten Dijke et al., 1993c). Thus, the specificity of ligand binding is postulated to be more dependent on the type II receptors than on the type I receptors. Before the present study, ActR-II and ActR-IIB had been shown to bind only activin and inhibin. Inhibin is a weak competitor for activin binding. Other ligands, e.g., TGF-βs and BMP-4, do not bind to ActR-IIIs (Mathews and Vale, 1991; Attisano et al., 1992; Mathews et al., 1992). Similarly, TβR-II and DAF-4, a BMP type II receptor from *C. elegans*, had been shown to bind only the respective ligands (Lin et al., 1992; Estevez et al., 1993). In the present study we show that OP-1, a member of the BMP family, binds ActR-II in transfected COS-1 cells, although the binding affinity of OP-1 to ActR-II is two- to threefold lower than that of activin A. Moreover, OP-1 transduced the p3TP-Lux transcription signal through ActR-II together with type I receptors. These results indicate that ActR-II can act as a functional type II receptor for OP-1, as well as for activin.

OP-1 also bound to ActR-IIB, and formed complexes with ActR-I, BMPR-IA and BMPR-IB. However, OP-1 induced p3TP-Lux transcriptional activation less efficiently through the complex of ActR-IIB, and ActR-I, compared to those of ActR-II and ActR-I. In contrast, activin A was shown to efficiently activate p3TP-Lux transcription through ActR-IIB, and ActR-I (Attisano et al., 1993).

In the presence of ActR-II, activin A binds ActR-I and ActR-IB, whereas OP-1 binds ActR-I, BMPR-IA and BMPR-IB, but not ActR-IB. These results suggest that the binding specificity of type I receptors is not solely determined by type II receptors, but type I receptors appear to recognize complexes of ligands and type II receptors. It was recently suggested that type I receptors specify the signals after ligand stimulation (Cárcamo et al., 1994). Thus, after the stimulation by activin A, ActR-I was found to mediate p3TP-Lux transcription, whereas ActR-IB transduced signals for p3TP-Lux transcription, growth inhibition and PAI-1 production. Although we observed no differences between ActR-1 and BMPR-IB in the functional assays investigated, they may possibly differ in the transduction of certain other signals.

We showed that both activin A and OP-1 inhibit [H]thymidine incorporation in Mv1Lu cells with similar...
potency. Moreover, the growth inhibitory activity of OP-1 was neutralized by follistatin, a specific inhibitor for activins (Ueno et al., 1987; Nakamura et al., 1990). However, whereas follistatin inhibits activin A at equimolar amounts, a 10-fold excess was needed to inhibit OP-1. Follistatin is a monomeric glycoprotein, that is expressed in parallel with activins and is suggested to modulate their functions. These results suggest that OP-1 and activin A, having 43% amino acid sequence identity (Ozkaynak et al., 1999), share certain structural properties important for the interaction with receptors as well as with follistatin.

Since OP-1 and activin have overlapping receptor binding specificities, it was interesting to compare the biological effects of OP-1 with those of activin. OP-1 induced erythroid differentiation in K562 cells, but the activity was lower than that of activin A. We showed that both OP-1 and activin A bind to ActR-II and ActR-I in K562 cells, whereas activin A, but not OP-1, binds to ActR-IB in these cells. Recently, K562 cells were found to express both ActR-II and ActR-IB mRNAs (Hildén et al., 1994). Since erythroid differentiation was more significant by activin A than by OP-1, it is possible that ActR-IB or other receptors, which do not bind OP-1 efficiently, are important for erythroid differentiation in K562 cells.

In contrast to activin A, OP-1 did not show any FSH-releasing activity. The rat pituitary cells express ActR-II and ActR-IB, which were shown to bind activin A. OP-1 bound to ActR-II very weakly in these cells; however, no type I receptor was observed after binding and cross-linking of [125I]OP-1, since OP-1 did not bind to ActR-IB in the presence of ActR-IIs.

OP-1 has been shown to be a multifunctional protein that has biological activities for BMPs (Ozkaynak et al., 1999; Sampath et al., 1992). We have shown here that OP-1 binds ActR-II and ActR-IB, and transduces certain activin signals. Biological effects of OP-1, which are shared by activin A, included neutralization by follistatin and stimulation of erythroid differentiation. We have also shown that OP-1 is a poor inducer of mesoderm in Xenopus embryos. It has recently been shown that mesoderm induction by Vg1, another member in the BMP subfamily, was blocked by truncated ActR-IIs (Schulte-Merker et al., 1994). Thus, proteins in the BMP family have broader biological functions than previously realized. Studies on the binding of the different members in BMP family to different type II and type I receptors will be important to understand the full range of their biological effects. It will also be important to determine whether ActR-II, together with type I receptors, transduces signals that are important for bone morphogenesis in vivo.

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