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Characterization and Cloning of a Receptor for BMP-2 and BMP-4 from NIH 3T3 Cells

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The bone morphogenetic proteins (BMPs) are a group of transforming growth factor β (TGF-β)-related factors whose only receptor identified to date is the product of the *daf-4* gene from *Caenorhabditis elegans*. Mouse embryonic NIH 3T3 fibroblasts display high-affinity ^{125}I-BMP-4 binding sites. Binding assays are not possible with the isoform ^{125}I-BMP-2 unless the positively charged N-terminal sequence is removed to create a modified BMP-2, ^{125}I-DR-BMP-2. Cross-competition experiments reveal that BMP-2 and BMP-4 interact with the same binding sites. Affinity cross-linking assays show that both BMPs interact with cell surface proteins corresponding in size to the type I (57- to 62-kDa) and type II (75- to 82-kDa) receptor components for TGF-β and activin. Using a PCR approach, we have cloned a cDNA from NIH 3T3 cells which encodes a novel member of the transmembrane serine/threonine kinase family most closely resembling the cloned type I receptors for TGF-β and activin. Transient expression of this receptor in COS-7 cells leads to an increase in specific ^{125}I-BMP-4 binding and the appearance of a major affinity-labeled product of ~64 kDa that can be labeled by either tracer. This receptor has been named BRK-1 in recognition of its ability to bind BMP-2 and BMP-4 and its receptor kinase structure. Although BRK-1 does not require cotransfection of a type II receptor in order to bind ligand in COS cells, complex formation between BRK-1 and the BMP type II receptor DAF-4 can be demonstrated when the two receptors are coexpressed, affinity labeled, and immunoprecipitated with antibodies to either receptor subunit. We conclude that BRK-1 is a putative BMP type I receptor capable of interacting with a known type II receptor for BMPs.

The transforming growth factor β (TGF-β) superfamily contains a large number of growth, differentiation, and morphogenetic cytokines that are active as homo- or heterodimers. This superfamily includes the TGF-β family, the activin family, the Müllerian inhibiting substance (MIS), and the bone morphogenetic protein (BMP)/Vg family, which composes the largest group (reviewed in reference 39). Human BMP-2 and BMP-4 and their *Drosophila* homolog dpp are highly related (74 to 76% sequence identity [72]), as are BMP-5, BMP-6, and BMP-7 and their *Drosophila* homolog 60A (69 to 73% sequence identity [21]). Given the high degree of structural similarity among these family members, it is expected that their receptors will also form a family of related molecules.

Binding proteins of various sizes have been identified for members of the TGF-β family by using affinity-labeling procedures and subsequent electrophoretic separation. Binding proteins of approximately 65 and 85 to 110 kDa (including the mass of the cross-linked radioligand) have been identified for TGF-β, activin, inhibin, BMP-4, and MIS in a variety of cell lines (12, 36, 38, 40, 47, 50). These proteins are referred to as type I and type II receptors, respectively, and their involvement in signaling was first inferred from their disruption in cell mutants selected for resistance to TGF-β (reviewed in reference 38).

Mammalian type II receptor cDNAs for TGF-β (35, 65) and activin (2, 40) have been cloned. It has recently been determined that the product of the *daf-4* gene from *Caenorhabditis elegans*, which is involved in both inhibition of dauer larva formation and exit from the dauer stage, is a type II receptor for BMP-2 and BMP-4 (24). Each of these receptors is predicted to be a transmembrane serine/threonine kinase. Ligand binding to each of these type II receptors in a variety of cell types does not appear to require coexpression of additional receptor components, indicating that type II receptors are capable of binding ligand on their own (75). In contrast, the type I receptors for TGF-β and activin are unable to bind ligand unless they are coexpressed with the corresponding type II receptor (1, 6, 11, 22, 23, 25, 57, 66, 76). The sequences of these type I receptors indicate that they, too, are predicted to be receptor serine/threonine kinases. While the kinase domains of the type I and type II receptors are more closely related to each other than to other protein kinase families, they nevertheless contain sequence motifs that distinguish them from each other. Moreover, the distinctive arrangement of cysteine residues in the extracellular domains of these receptors further suggests that these two receptor subunits exhibit distinct structural topologies. Expression of TGF-β receptor types I and II in the same cell is required for signalling (reviewed in reference 38), and both receptors form a tight complex in the presence of ligand (1, 11, 22, 25, 32, 57). These observations support a model in which TGF-β signals transcriptional and antimitogenic responses via a heteromeric kinase receptor complex (6, 11, 25, 75).

In contrast to what is known about the TGF-β and activin receptor systems, little is known about BMP receptors. Prelim-

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inary cross-linking experiments have suggested that BMPs may interact with proteins of sizes similar to the type I and type II receptors (2, 36, 41, 47). There is conflicting evidence (compare references 36, 41, and 47 with reference 2) concerning the existence of BMP-binding proteins of higher molecular weight that might be analogous to the TGF-β type III accessory receptors (38). In this paper, we present a characterization of endogenous BMP-2- and BMP-4-binding proteins in NIH 3T3 cells and demonstrate that these two ligands interact with the same cell surface proteins. Using the PCR cloning approach, we have identified a novel member of the transmembrane serine/threonine kinase receptor family that binds BMP-2 and BMP-4 with high affinity and whose sequence most closely resembles that of the previously cloned type I receptors for TGF-β and activin (1, 6, 11, 23, 25, 57, 66, 76). This novel receptor protein has been named BRK-1 to acknowledge its BMP-binding properties and its receptor kinase structure. In contrast to the TGF-β and activin type I receptors, this receptor does not require coexpression with an exogenous type II receptor in order to bind BMPs in COS cells. We also demonstrate that this putative BMP receptor is capable of forming a heteromeric complex with the DAF-4 BMP type II receptor.

MATERIALS AND METHODS

Production and purification of recombinant BMPs. Oligonucleotide probes for human BMP-2 (GCG GCC CGA CGA CGC CGC CGC GAA CTT CCT GCG GCC CAG CTC CGG A) and human BMP-4 (CTC CTG ATA ATT TTT CAG TAC CAC CTT ATC ATA CTC ATC CAG GTA) were labeled at the 5′ end with ^{32}P. Screening a selection of human cell lines by Northern (RNA) analysis with these probes demonstrated that BMP-2 mRNA and BMP-4 mRNA were present in HeLa D98 and U2OS cells, respectively. Blots were hybridized in a solution of 50% deionized formamide, 5× SSPE (1× SSPE is 0.18 M NaCl, 10 mM $NaPO_4$, and 1 mM EDTA [pH 7.7]), 2× Denhardt's solution, and 200 μg of salmon testis DNA per ml containing 10^6 cpm of probe per ml for 48 h at 37°C. Blots were washed in 0.25× SSPE–0.1% sodium dodecyl sulfate (SDS) at 37°C three times for 15 min each. cDNA libraries of RNA isolated from HeLa D98 and U2OS cells in λZAP (Stratagene, La Jolla, Calif.) were screened with the same oligonucleotide probes as above, under identical conditions. cDNAs encompassing the complete coding region were isolated and sequenced. They were identical in the coding region to the reported sequence (74).

Human BMP-2 (nucleotides −7 to 1231) and BMP-4 (nucleotides −8 to 1298) cDNAs were inserted into the pDSRα vector to generate expression plasmids pDSRα-BMP2 and pDSRα-BMP4. The pDSRα vector is a derivative of pcDL-SRα296 (kindly provided by DNAX Research Institute, Palo Alto, Calif.) which utilizes the simian virus 40 (SV40) early promoter in conjunction with the R element of human T-cell leukemia virus (HTLV) for high-level expression (58). The vector also contains the mouse dihydrofolate reductase minigene (17) for stable selection and gene amplification. To achieve high levels of BMP production, expression plasmids containing BMP-2 or BMP-4 cDNA were stably introduced into dihydrofolate reductase-deficient CHO (DG44) cells (67) and amplified to a high copy number by methotrexate selection. Cells producing high levels of BMP-2 or BMP-4 were identified by either Northern or Western (immunoblot) analysis.

Recombinant BMPs were purified from serum-free media conditioned with transfected CHO cells by methods analogous to those described previously (29, 53, 71). The media were concentrated by ultrafiltration and then chromatographed in succession over heparin-agarose, Zn^{2+}-chelating Sepharose, and C4 reversed-phase columns. An additional gel filtration step was necessary for purification of BMP-4. BMP-2 (molecular weight, 31,400) and BMP-4 (molecular weight, 32,800) were found to be greater than 95% pure as indicated by Coomassie blue and silver staining of SDS-polyacrylamide gels and by radiolabeling studies. Recombinant BMPs were quantitated by amino acid analysis, and their amino termini were verified by protein sequencing. Activity of the recombinant BMPs was assessed by using the BMP-mediated induction of bone in vivo, determined essentially as described by Reddi and Huggins (51).

Preparation of DR-BMP-2. Recombinant BMP-2 (rBMP-2) (100 to 250 μg) was solubilized in 500 μl of 4 M urea–0.1 M NaCl–0.05 M Tris-HCl (pH 8.2). Trypsin (sequencing grade; Boehringer Mannheim, Indianapolis, Ind.) was added to a trypsin/rBMP-2 ratio of 1/50 (wt/wt), and the digestion mixture was incubated at 37°C for 2 h. Digestion was stopped by the addition of phenylmethylsulfonyl fluoride (PMSF) to a final concentration of 1 mM. "Digit-removed" BMP-2 (DR-BMP-2) was purified from the digestion mixture by reversed-phase high-pressure liquid chromatography (HPLC) on a Waters Delta-Pak C_4 column (5 μm, 300 Å [1 Å = 0.1 nm], 3.9 by 150 mm). The majority of DR-BMP-2 eluted as a well-defined peak at about 0.05% trifluoroacetic acid and 36% acetonitrile, as monitored by A_{214} and after Coomassie blue staining of an SDS-polyacrylamide gel. PMSF, PMSF-inactivated trypsin, and any remaining intact rBMP-2 were separated from DR-BMP-2 under these chromatographic conditions. Purified DR-BMP-2 was aliquoted, dried under a vacuum, and stored at −20°C.

Analysis by SDS-polyacrylamide gel electrophoresis (PAGE) showed that partial digestion with trypsin decreased the molecular mass of nonreduced rBMP-2 by about 2,000 Da and reduced rBMP-2 by about 1,000 Da. Amino-terminal protein sequencing showed that roughly 70% of DR-BMP-2 begins at Lys-290, while the remaining 30% begins at Leu-292. Results from amino acid analysis were entirely consistent with the sequencing results and suggested that the carboxy terminus of the protein was unaffected by trypsin treatment.

Isolation and characterization of cDNA clones. PCR primers were designed to encompass the regions in kinase domain II to kinase domain VIB containing an insert that is characteristic of the TGF-β superfamily of receptor kinases and absent in other protein kinase families. The 5′ primer pool was composed of two degenerate oligonucleotides [Act 2A, 5′-GA(AG) GCI GTI GCI GTI AA(AG) (AG)TI TT-3′, and Act 2B, 5′-GA(AG) TA(TC) GTI GCI GTI AA(AG) (AG)TI TT-3′], designed to encode the protein sequence E A/Y V A V K V/I F, which is found in kinase domain II of the activin and DAF-1 receptors. The 3′ primer pool was composed of two degenerate oligonucleotides [Act 1A, 5′-TT(AG) AT(AG) TCI C(TG)(AG) TG(AG) CTI ATI GCI GG(TC) TT-3′, and Act 1B, 5′-TT(AG) AT(AG) TCI C(TG)(AG) TGI G(AC)I ATI GCI GG(TC) TT-3′], derived from the antisense strand corresponding to the protein sequence K P A M/I A/S H R D I K, which is found in domain VIB of the activin and DAF-1 receptors. An *Eco*RI site was inserted at the 5′ end of each primer to facilitate subcloning.

To prepare a template for PCR, 200 ng of poly(A)$^+$ RNA isolated from NIH 3T3 cells was reverse transcribed to single-stranded cDNA. PCR was performed on 20% of this material, with 50 pmol of each of the 5′ primers (Act 2A and Act 2B) and 250 pmol of each of the 3′ primers (Act 1A and Act 1B)

in a 100-μl final volume under standard PCR conditions (94°C for 2 min followed by 35 cycles of 94°C for 30 s, 55°C for 30 s, and 72°C for 30 s). A 10-μl aliquot of the reaction product was removed and subjected to another 35 cycles of amplification with fresh reagents. Products of the secondary PCR were ligated to pCR1000 (Invitrogen, San Diego, Calif.) for clonal selection and sequence analysis. One clone, J159-PCR, appeared to encode a portion of a novel receptor kinase.

To isolate the full-length cDNA clone, a λZAPII NIH 3T3 cDNA library (Stratagene) was screened with J159-PCR. Hybridization was performed for 24 to 48 h at 42°C in 5× SSPE–1× Denhardt's solution–100 μg of salmon testis DNA per ml–50% formamide. Membranes were washed first at 42°C and subsequently at 55°C in 0.1× SSPE and 0.2% SDS. Rescreening of positive plaques yielded two clones, one of which (J159#7) was renamed *BRK-1T*. Sequence analysis demonstrated a shortened kinase domain in this clone compared with the sequences of other known receptor kinases. An additional NIH 3T3 cDNA library, prepared in UNI-ZAP with an oligo(dT) and a random hexanucleotide primer for the synthesis of cDNA (Stratagene), was screened with both the PCR product and the J159#7 cDNA to obtain seven more clones. One of these clones, J159#P12B, contained the complete uninterrupted coding sequence and was renamed *BRK-1*.

COS cell expression. The *BRK-1* and *daf-4* expression plasmids were constructed by inserting the *BRK-1* cDNA (nucleotides −10 to 1791) and *daf-4* cDNA (24) (nucleotides −41 to 2329) into the multiple cloning sites of the pJT4 vector. The pJT4 vector, which was designed for high-level transient expression of cDNAs, contains the cytomegalovirus early promoter/enhancer, the splice and poly(A) signals of SV40, and the R-U5 segment of HTLV type 1 (HTLV-1) (58). The origin of replication from SV40 was also included in the vector to allow autonomous replication of the plasmid in the COS cells.

COS cells were cultured in Dulbecco's modified Eagle medium containing 5% fetal bovine serum. COS-7 cell aliquots containing 5×10^6 cells were transiently transfected with 10 μg of *BRK-1*, 20 μg of *daf-4*, or both expression plasmid cDNAs by electroporation (Bio-Rad Gene Pulser) at 4.0 kV/cm, with a capacitance of 25 μF. Transfected cells were seeded at 10^7 cells per 10-cm-diameter plate for immunoprecipitations or distributed equally among the wells in 12-well plates for cross-linking and binding experiments. After 24 h, attached cells were washed with phosphate-buffered saline and fresh media were added. Forty eight hours posttransfection, cells were assayed for BMP binding. Transfection of COS-1 cells was as previously described (1).

BMP receptor binding and cross-linking. The BMPs were iodinated by the chloramine T procedure as previously described (26). Typically, the specific activity ranged from 3,000 to 8,000 Ci/mmol and 3,000 to 15,000 Ci/mmol for ^{125}I-BMP-4 and ^{125}I-DR-BMP-2, respectively. The lactoperoxidase method of iodination is not suitable for iodination of the BMPs because this method results in the formation of aggregates that are not susceptible to reduction by either dithiothreitol or β-mercaptoethanol (data not shown). Presumably, the aggregates are the result of alkylation of tyrosine residues via ether linkages, as has been demonstrated in other systems with this enzyme (62).

Confluent NIH 3T3 cells or transfected COS-7 cells were affinity labeled with ^{125}I-BMP-4 or ^{125}I-DR-BMP-2 by established procedures (37), except that a protease inhibitor cocktail (10 μg of leupeptin per ml, 10 μg of antipain per ml, 50 μg of aprotinin per ml, 100 μg of benzamidine per ml, 100 μg of soybean trypsin inhibitor per ml, 10 μg of bestatin per ml, 100 μg of pepstatin A per ml, 0.3 mM PMSF) was included during both the binding and the cross-linking procedures. The addition of protease inhibitors during binding decreased nonspecific binding (data not shown); 5 to 30 nM BMP-4 or BMP-2 was used to determine nonspecific binding. For affinity labeling studies, radiolabeled BMP was chemically cross-linked to the receptor with 0.135 mM disuccinimidyl suberate (DSS) as described elsewhere (37). Solubilized cell proteins were separated on SDS–7.5% polyacrylamide gels.

Preparation of antisera and immunoprecipitation. Two antisera against BRK-1 were raised in rabbits (Hazleton Washington, Vienna, Va.). One was directed against the extracellular domain (ECD) containing amino acids 24 to 152 of BRK-1 expressed in *Escherichia coli*, using the QIA Express system (Qiagen, Chatsworth, Calif.). The ECD protein was purified from inclusion bodies over a nickel-nitrilotriacetic acid resin according to the manufacturer's instructions. Purification was completed by reversed-phase HPLC on a Waters DeltaPak C4 column (300 Å, 7.8 mm by 30 cm). The second antiserum was directed against an HPLC-purified synthetic peptide corresponding to amino acids 398 to 420 of BRK-1, conjugated to keyhole limpet hemocyanin via a cysteine added to the C terminus.

Affinity-labeled endogenous BRK-1 in NIH 3T3 cells, or BRK-1 expressed in COS cells, was solubilized and immunoprecipitated with either antibody as previously described (24, 63). Immune complexes were pelleted with Pansorbin, sample buffer was added, and solubilized proteins were separated on SDS–7.5% polyacrylamide gels.

Northern analysis. A mouse tissue blot containing poly(A)$^+$ RNA isolated from adult BALB/c mice (Clontech, Palo Alto, Calif.) was probed with a random primed, labeled 0.4-kb *Nco*I fragment encoding the extracellular domain of BRK-1. Hybridization and washing of the blot were performed under the conditions recommended by the vendor.

Sequence analysis. The sequence analysis was performed by using the FPATTERNALIGN and TREEANALYSIS suite of programs (15). The FPATTERNALIGN program is based on the flexible pattern alignment procedure of Barton and Sternberg (4). This has been adapted to use patterns that include entries that represent common groups of amino acids. An initial pattern for the protein kinases was derived from the alignment of Hanks and Quinn (30) and refined after initial alignment of a subset of the known kinases. An example of the sequence alignment is illustrated in Fig. 4A. The logcounts similarity array (15) was derived from multiple sequence alignments of proteins in the Protein Data Bank (7).

The TREEANALYSIS involves using the pattern derived above to fix the alignment of conserved domains and individually align the intervening segments (15). Patterns were found for domains I to IX in protein kinases, but no consistent pattern could be found for domains X and XI across the entire protein kinase database. The TREEALIGN program aligns each sequence to the pattern and stores the results in binary pointer arrays. TREEADJUST is used to correct misalignments of individual sequences to the pattern. TREESCORE calculates the cross scores for all pairs of sequences individually for the segments between the selected fixed domains. This is accomplished by using a standard Needleman-Wunsch algorithm (45). The alignment can be started and stopped at fixed points relative to the first and last domains selected for analysis. This enables an implied analysis of domains X and XI by cutting the analysis off at a length that approximates the residues needed to include these two domains (80 residues). Finally, the similarity tree (see Fig. 4B) is generated by the TREEGEN program. This starts with the highest self-scoring sequence and proceeds to add sequences to the family by

TABLE 1. Comparison of relative binding affinities in NIH 3T3 cells

Competitor	^{125}I-BMP-4[a]			^{125}I-DR-BMP-2[a]		
	Log IC_{50} ± SEM	IC_{50} (M)	*n*	Log IC_{50} ± SEM	IC_{50} (M)	*n*
BMP-4	−10.400 ± 0.061	3.98×10^{-11}	3	−9.752 ± 0.285	1.77×10^{-10}	2
BMP-2	−9.797 ± 0.344[b]	1.60×10^{-10}[b]	4	−9.572 ± 0.925	2.68×10^{-10}	2
DR-BMP-2	−10.776 ± 0.191	1.68×10^{-11}	4	−9.528 ± 0.037	2.96×10^{-10}	2

[a] ^{125}I-BMP-4 was added at a concentration of 60 to 130 pM. ^{125}I-DR-BMP-2 was added at a concentration of 60 to 70 pM.
[b] Does not significantly differ from either BMP-4 ($P = 0.2041$) or DR-BMP-2 ($P = 0.0621$).

including the sequence with the highest score relative to the present set and above a given cutoff (typically 85%). This process is repeated until all sequences are assigned to families. The families are then ranked relative to the family containing the sequence of interest by calculating the average cross score between all members of the two families.

Data analysis. The saturation isotherms were modeled by using the mass action equation with the LIGAND program (44), adapted for use on an MS-DOS computer (Elsevier Biosoft, Ferguson, Mo. [43]). Competition curves were modeled by using the four-parameter logistic function with the INPLOT program package (Graph Pad Software, San Diego, Calif.), version 4.0. Each competition curve was modeled independently. For the saturation experiments, errors in parameter estimates are provided in the tables as approximate standard errors in the estimate, as determined by the analysis. In the case of the competition experiments, the significance in the difference in the parameter estimates for the different BMP analogs (Table 1) was determined with the SAS package by performing the *F* test for randomized block design (28) on a DEC VAX 8530 minicomputer. The LIGAND and INPLOT programs were run on a Gateway 2000 (486DX/33) personal computer.

Nucleotide sequence accession number. The sequence of *BRK-1* has been deposited in GenBank under accession number U04672. The sequence of *BRK-1T* has been deposited in GenBank under accession number U04673.

RESULTS

Characterization of the BMP-binding proteins in NIH 3T3 cells. As a first step to characterize BMP receptors present in NIH 3T3 cells, we determined that equilibrium binding at 4°C to NIH 3T3 monolayers was achieved within 4 h and maintained for at least 1 h thereafter (data not shown). ^{125}I-BMP-2 and ^{125}I-BMP-4 showed different binding properties under these equilibrium binding conditions. ^{125}I-BMP-4 bound specifically, as determined by the ability of unlabeled ligands to compete (Fig. 1). Nonspecific binding, defined as the residual binding obtained in the presence of 10 nM unlabeled BMP-2 or BMP-4, typically ranged from 40 to 70% of the total binding in whole-cell binding assays (Fig. 1). In contrast, ^{125}I-BMP-2 binding was not decreased by the presence of excess unlabeled BMP-2 or BMP-4, making assessment of specific binding to receptor proteins impossible to define with this tracer. This problem might relate to the strong tendency of BMP-2 to adhere nonspecifically to culture dishes or to the negatively charged extracellular matrix via its positively charged N terminus (5 of the first 9 residues are lysine or arginine). Therefore, we modified BMP-2 by mild trypsin digestion (see Materials and Methods) to produce a form, named DR-BMP-2, that lacks the first seven to nine N-terminal residues. ^{125}I-DR-BMP-2 displayed specific binding to NIH 3T3 cells (Fig. 1). In subsequent studies, ^{125}I-DR-BMP-2 was used in order to characterize BMP-2 receptors and to establish that BMP-2 and BMP-4 interact with the same receptor proteins in NIH 3T3 cells.

Analysis of the ^{125}I-BMP-4 saturation binding isotherm (Fig. 2), by fitting it to a one-binding-site model with the LIGAND program (43, 44), yielded a K_d of $\sim 2.54 \times 10^{-10}$ M and a B_{max} of 4.11×10^{-12} M, which corresponds to ~1,400 binding sites per cell. The K_d value was ~10-fold higher than the 50% inhibitory concentration (IC_{50}) values obtained for BMP-4 in a self-competition assay (Table 1), suggesting that iodination may decrease the binding affinity of BMP-4. Interestingly, DR-BMP-2 and BMP-4 exhibited a lower IC_{50} when ^{125}I-BMP-4 was the tracer than when ^{125}I-DR-BMP-2 was the tracer (Table 1). Nevertheless, BMP-4, BMP-2, and DR-BMP-2 were similar in relative competing potency irrespective of whether ^{125}I-BMP-4 or ^{125}I-DR-BMP-2 was used as the tracer (Table 1), suggesting that the two radioligands label the same binding proteins.

To visualize BMP-binding proteins, ^{125}I-BMP-4 or ^{125}I-DR-BMP-2 was cross-linked to NIH 3T3 cells and affinity-labeled complexes were resolved by SDS-PAGE of cell lysates under reducing conditions (Fig. 3). With the molecular mass of the cross-linked ligand monomer substracted, both radioligands specifically labeled proteins of approximately 57 to 62 kDa and 75 to 82 kDa. These values correspond, respectively, to those of the type I and type II receptors for TGF-β and activin (1, 2, 25, 35, 38, 40). An affinity-labeled band of approximately 135 kDa (Fig. 3; more readily visible in cells labeled with ^{125}I-DR-BMP-2 than in those labeled with ^{125}I-BMP-4) might represent a heteromeric type I-type II complex or a distinct receptor

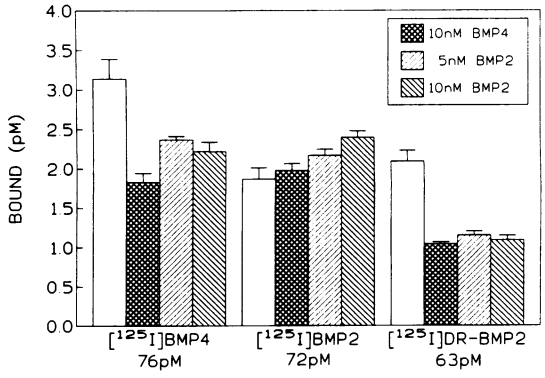


FIG. 1. Comparison of whole-cell binding properties of the various BMPs. ^{125}I-BMP-4 (76 pM), ^{125}I-BMP-2 (72 pM), and ^{125}I-DR-BMP-2 (63 pM) were incubated with NIH 3T3 cell monolayers at 4°C for 4, 3.5, and 4 h, respectively. The data represent the averages of triplicate determinations ± standard errors of the mean determined in the absence (blank bars) or presence of the indicated competitors. Similar results were obtained in three additional experiments.

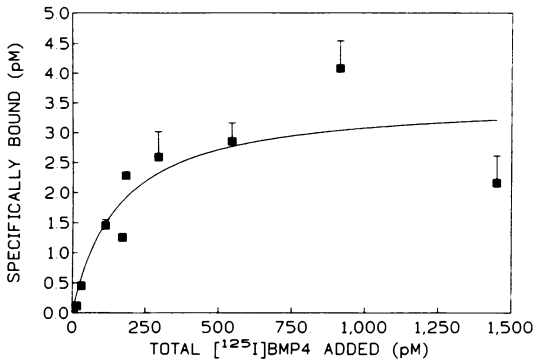


FIG. 2. Saturation isotherm for ^{125}I-BMP-4 binding in NIH 3T3 cells. The datum points represent the averages of triplicate determinations ± standard errors of the mean. The line represents the fit of the data to a one-binding-site model. The fitted curve parameters, obtained by using LIGAND, are as follows: $K_d = 2.54 \times 10^{-10}$ M and $B_{max} = 4.11 \times 10^{-12} \pm 0.99 \times 10^{-12}$ M, where the error represents the approximate standard error in the parameter estimate. Specific binding, defined as the difference in levels of binding obtained in the presence and in the absence of 10 nM BMP-4, is shown. Nonspecific binding composed approximately 50% of the total signal in the region of the K_d.

species. BMP-2, BMP-4 and DR-BMP-2 effectively competed for binding to the proteins labeled by either ^{125}I-BMP-4 or ^{125}I-DR-BMP-2, whereas TGF-β_1 and -β_2 had no effect (Fig. 3). This, together with the observation that both tracers label proteins of similar size, suggests that BMP-2 and BMP-4 interact with the same receptor proteins in NIH 3T3 cells. Furthermore, we do not detect binding to a high-molecular-weight protein with the characteristics of the type III accessory receptor for TGF-β (38).

Cloning of a novel member of the serine/threonine kinase receptor family. NIH 3T3 cells were used as the source of mRNA to clone members of this receptor family by PCR. Alignment of the amino acid sequences for the kinase domains of the orphan receptor DAF-1 (27) and the mouse activin type II receptor ActR-II (40) with that of the cytosolic Raf kinase (9, 46) revealed the presence of a unique insert in the receptor kinases, located between kinase subdomains VIA and VIB. In order to ensure that we amplified kinases that would contain this insert, primers were designed to amplify regions of sequence between kinase subdomains II and VIB (see Materials and Methods). An insert of approximately 300 bp that exhibited homology to ActR-II and DAF-1 was isolated. Screening of two NIH 3T3 cDNA libraries with this PCR insert yielded nine clones. Sequencing revealed that one of these clones contained a 2,402-bp insert with an open reading frame of 1,596 bp starting with an ATG codon at bp 11 that is in a favorable context for translation initiation (34). This clone was designated *BRK-1*. Another clone corresponding to the same species contains an additional 280 nucleotides of the 5′ untranslated region and what appears to be an incompletely processed intron interrupting the kinase region (data not shown). This clone was designated *BRK-1T*. Interestingly, the 90-bp insertion present in *BRK-1T* results in the insertion of an in-frame stop codon in kinase domain XI, after amino acid 491. This truncation occurs in a location similar to that observed for the splice variants of SKR-2 (77), which corresponds to variants of the activin type IB receptor (11) and to a variant of the BMP type II receptor DAF-4 (24). The presence of an intron-exon boundary in a similar location in the mouse activin type II receptor gene (42) and the *daf-1* (27) and SKR-2 (77) receptor genes makes it tempting to speculate that the *BRK-1T* clone might represent a truncation of the full-length BRK-1 receptor. Determination of the *BRK-1* gene structure and verification of the expression of the truncated receptor protein are necessary before the relevance of this clone can be ascertained.

The *BRK-1* open reading frame encodes a protein of 532 amino acids (predicted molecular mass, 60.1 kDa) with the predicted structure of a transmembrane serine/threonine kinase (Fig. 4A). The putative N-terminal signal sequence contains a potential cleavage site between amino acids 23 and

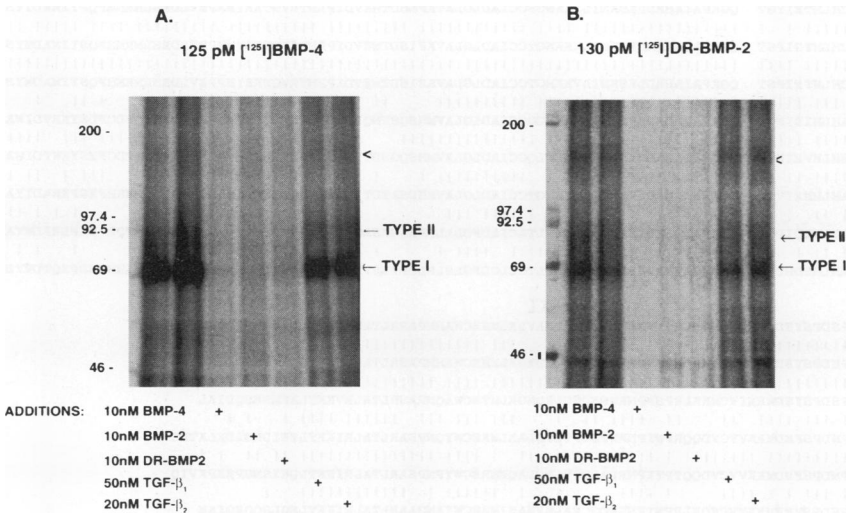
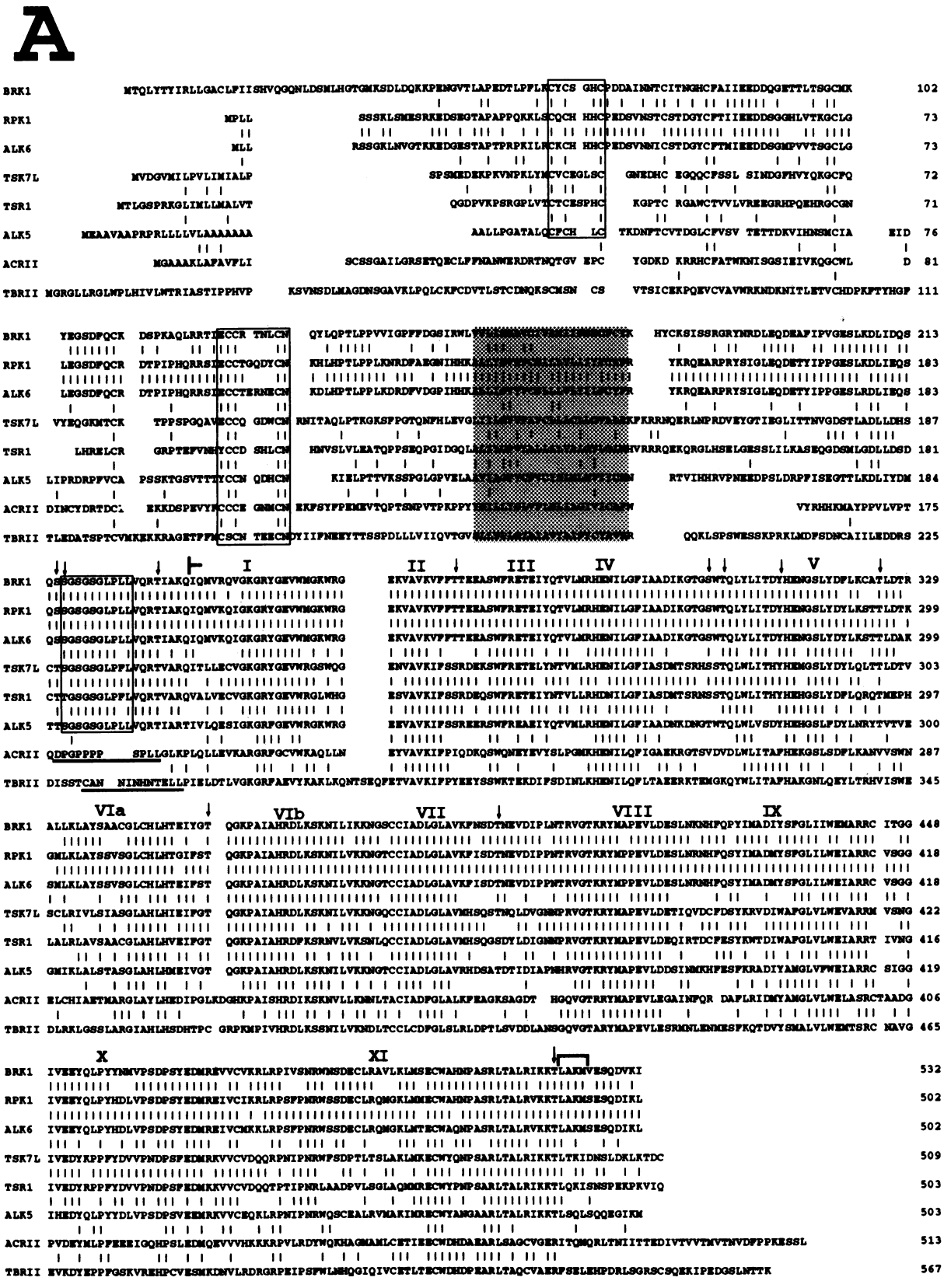


FIG. 3. Affinity labeling of BMP-binding proteins in NIH 3T3 cells. Cells were affinity labeled with ^{125}I-BMP-4 (A) or ^{125}I-DR-BMP-2 (B) in the presence and absence of the indicated ligands and then chemically cross-linked to the receptor with 0.135 mM DSS. The labeled cell extracts were subjected to SDS-PAGE on 7.5% polyacrylamide gels. The autoradiograph was visualized with a PhosphorImager (Molecular Dynamics). Numbers on the left of each panel represent the molecular mass (in kilodaltons) markers. Similar results were obtained in three independent experiments. The higher-molecular-mass band with a molecular mass of ~135 kDa after subtraction of the molecular mass of the ligand monomer is indicated by <.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BRK1
RPK1
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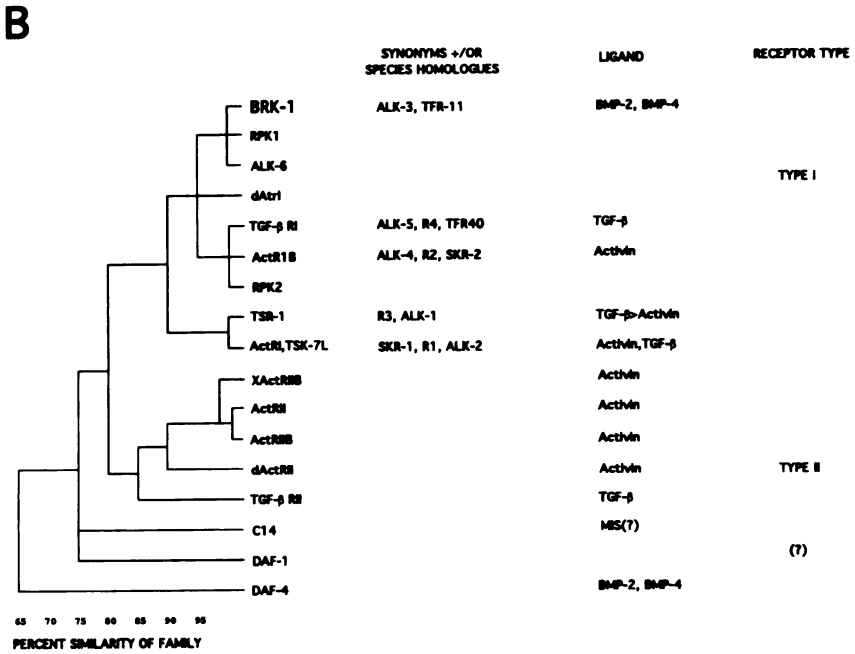

FIG. 4. Comparison of BRK-1 with previously cloned type I and type II receptors. (A) Sequence alignment of BRK-1 with other receptor serine/threonine kinases. RPK1, a chicken type I receptor (56); ALK-6, a mouse type I receptor (61); ActR1, the mouse activin/TGF-β type I receptor TSK7L (22, 23); TSR1, a human type I receptor for TGF-β with unknown function (1); ALK-5, a mouse type I receptor for TGFβ (25); ACRII, mouse activin type II receptor (40); TBRII, rat TGF-β type II receptor (65). The start of the kinase domain is indicated by ⊢, and the end of the kinase domain is in the region indicated by the bracket (30), as the substrate specificity of this kinase remains unknown. The shaded box represents the putative transmembrane domain. The open boxes in the extracellular domain highlight the upstream and downstream cysteine boxes. The open box in the cytoplasmic juxtamembrane region highlights the SGSG motif that is present in the type I receptors and absent in the type II receptors. The arrows indicate S/T residues that are unique to the type I receptors. (B) Kinase domain similarity dendrogram for receptors for members of the TGF-β superfamily. The dendrogram was created by running the TREEGEN program with different cutoffs for the formation of groups. Vertical lines in the dendrogram correspond to the percent cutoff level, on the scale at the bottom, at which the families combine. The cutoff was varied from 60 to 95% in 5% increments. The 98% cutoff represents combination at 98% or above.

24 (68). The single putative transmembrane region encompasses residues 155 to 176. A potential site of N glycosylation is found at residue 73 in the relatively short extracellular region. This region contains a cluster of cysteine residues (cysteine box) located within 30 amino acids upstream of the transmembrane region characteristic of all known members of the mammalian serine/threonine kinase receptor family (Fig. 4A). This motif is also present in the reverse orientation in various receptor serine/threonine kinases from plants (55, 64, 69, 70). In addition, there is an upstream cysteine box at amino acid residues 61 to 67 that is characteristic of previously cloned type I receptors for activin and TGF-β (1, 6, 11, 23, 25, 57, 66, 76) but is not present in any of the type II receptors cloned to date (Fig. 4A and data not shown).

BRK-1 contains the sequence SGSGSGLP followed by two hydrophobic residues in the juxtamembrane region, just upstream of the kinase domain, which is present in the other type I receptors. The internal juxtamembrane region also contains consensus sites for phosphorylation by protein kinase C (PKC; residues 184 and 205), casein kinase II (residue 205), and cyclic AMP-protein kinase (cAMP-PK; residue 181). Within the kinase domain, Thr-400 is located in the putative autophosphorylation loop between kinase subdomains VII and VIII and aligns with Thr-197 in cAMP-PK (59) and Tyr-185 in mitogen-activating protein kinase (16), residues whose phosphorylation increases catalytic activity of those kinases. Thr-391 in BRK-1 aligns with a Ser or Thr in other type I receptors (Fig. 4A) and might also represent an activating phosphorylation site. Additional potential phosphorylation sites for cAMP-PK, casein kinase II, and PKC are located in kinase subdomains X and XI and the carboxy terminus at residues 443, 486, and 498 (cAMP-PK); 466, 489, and 527 (casein kinase II); and 484 (PKC).

Kinase subdomains VIB and VIII in BRK-1 contain sequences characteristic of serine/threonine kinases (30). The BRK-1 kinase domain is most similar to those of type I receptors for activin (1, 11, 22, 66, 76) and TGF-β (1, 6, 25, 57) as well as a variety of related orphan receptors (31, 60) (Fig. 4). The human ALK3 orphan receptor (60), the chicken RPK1 orphan receptor (56), and the mouse ALK-6 orphan receptor (61) are the members of this family most closely related to BRK-1. Over the entire sequence, these receptors exhibit 98.3, 66.7, and 66.9% sequence identities, respectively, with the sequence identity based on the length of BRK-1.

BRK-1 binds BMP-4 and BMP-2. Mock-transfected COS-7 cells showed a small amount of specific ^{125}I-BMP-4 binding, indicating that these host cells express endogenous BMP-4 receptors (Fig. 5A). Transfection of *BRK-1* increased the specific binding of ^{125}I-BMP-4 to COS-7 cells (Fig. 5A). Since BRK-1 structurally resembles the type I receptors for TGF-β and activin and these receptors require coexpression with a type II receptor in order to bind ligand (1, 6, 11, 22, 23, 57, 66, 75, 76), we were surprised to detect increased BMP binding to *BRK-1*-transfected cells in the absence of a cotransfected type II receptor cDNA. However, given the presence of endogenous BMP receptors in COS-7 cells, it is possible that trans-

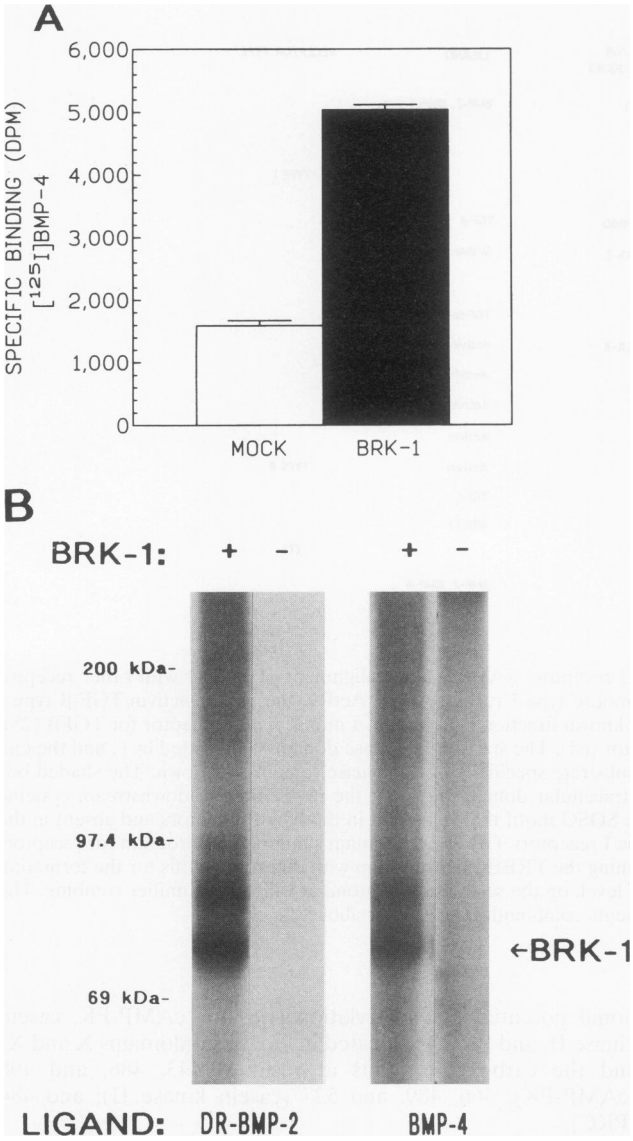

FIG. 5. Transfection of BRK-1 increases ^{125}I-BMP-4 binding. (A) Increase in ^{125}I-BMP-4-specific binding in *BRK-1*-transfected COS-7 cells. Transient transfection of 10 μg of *BRK-1* per 5×10^6 COS-7 cells is compared with 10 μg of vector alone (MOCK) per 5×10^6 COS-7 cells. Whole-cell binding was performed with 100 pM ^{125}I-BMP4 in the absence or presence of 10 nM unlabeled BMP4 for nonspecific binding. (B) Affinity labeling of ^{125}I-BMP-4 and ^{125}I-DR-BMP-2 to *BRK-1*-transfected COS-7 cells. COS-7 cells transfected with BRK-1 (+) or vector alone (−) were affinity labeled with either 100 pM ^{125}I-BMP-4 or 50 pM ^{125}I-DR-BMP-2. After a cross-linking with 0.135 mM DSS, solubilized proteins were analyzed by SDS-PAGE on 7.5% polyacrylamide gels. The positions of affinity-labeled BRK-1 and molecular mass markers are indicated.

fected BRK-1 bound BMPs in concert with an endogenous BMP receptor.

Affinity labeling of *BRK-1*-transfected COS-7 cells with ^{125}I-BMP-4 or ^{125}I-DR-BMP-2 yielded a labeled product corresponding to a species of ~64 kDa after subtraction of the cross-linked ligand monomer and a less intensely labeled product corresponding to a 77-kDa species after subtraction of the molecular mass of the ligand monomer (Fig. 5B). As the predicted molecular mass of BRK-1 is ~60 kDa, it is likely that the lower of the two bands corresponds to transfected BRK-1; this is also supported by the results of immunoprecipitation experiments (see below). Since the molecular weight of the ^{125}I-BMP-4, as estimated from SDS gels, is 32,800 (data not shown), the species corresponding to the upper band might represent BRK-1 cross-linked to the ligand dimer. Alternatively, this band might correspond to an endogenous type II receptor present in the COS cells that becomes apparent upon overexpression of the type I receptor.

Since two of the type I receptors cloned to date have exhibited the ability to bind either activin or TGF-β in the presence of the appropriate type II receptor (1, 22), we determined whether BRK-1 would exhibit similar properties. Figure 6B and C illustrate that BRK-1 does not bind ^{125}I-TGF-β_1 or ^{125}I-activin on its own or when cotransfected with the respective type II receptor cDNA. The ability of BRK-1 to bind ^{125}I-BMP-4 in the presence of the TGF-β type II receptor (Fig. 6A) also indicates that the lack of ^{125}I-TGF-β_1 binding to BRK-1 is unlikely to be due to a decrease in binding caused by cotransfection of the TGF-β type II receptor (23, 66, 76). In addition, we were unable to observe ^{125}I-TGF-β_1 binding to BRK-1 when different ratios of BRK-1 to TGF-β type II receptor cDNA were used in the transient transfections (data not shown). Although activin has been demonstrated to compete for ^{125}I-BMP-4 binding sites under certain circumstances (36), we do not see evidence for activin binding to BRK-1 either on its own or in conjunction with the activin type II receptor (Fig. 6C).

Immunoprecipitation of BMP–BRK-1 complexes. To confirm that the BMP-labeled species are associated with the BRK-1 protein, polyclonal antibodies to both a recombinant BRK-1 extracellular domain and a synthetic peptide corresponding to amino acids 398 to 420 of BRK-1 were generated. The latter is a region located in a predicted loop between kinase subdomains VII and VIII (Fig. 4A). Separate immunoprecipitations of lysates from affinity-labeled COS-7 cells expressing BRK-1 yielded a labeled product corresponding to an ~62- to 66-kDa (after subtraction of the molecular mass of the ligand monomer) receptor species with both types of antisera (Fig. 7A). In addition, binding to the immunoprecipitated BRK-1 protein was effectively inhibited by 30 nM unlabeled BMP-4, BMP-2, or DR-BMP-2 (Fig. 7A) as is seen with the native receptor in NIH 3T3 cells (Fig. 3).

Both BRK-1 antisera also immunoprecipitated a series of proteins from NIH 3T3 cells (Fig. 7B) resembling those seen in the cross-linking experiments (Fig. 3). The molecular mass of the major protein immunoprecipitated by the BRK-1 antisera in NIH 3T3 cells is ~68 to 71 kDa (after subtraction of the molecular mass of the ligand monomer), similar to that observed with BRK-1 expressed in COS-7 cells. To address the possibility that the additional affinity-labeled bands (Fig. 7B) present in the NIH 3T3 BRK-1 immunoprecipitations were additional receptor subunits, the samples were boiled in 1% SDS prior to addition of the antibody in order to dissociate any noncovalently bound proteins. The higher-molecular-weight bands (also present in the affinity-labeled NIH 3T3 cells [compare Fig. 3 and 7B]) disappear in those samples which were boiled in SDS prior to immunoprecipitation with the BRK-1 antibody. The band corresponding in size to the type II receptor (compare Fig. 3 and 7B) does not disappear with SDS treatment, suggesting that it is covalently associated with BRK-1. The ability of two antibodies specific for different regions of the BRK-1 protein to immunoprecipitate an affinity-labeled protein, with the expected size of a type I receptor, from both NIH 3T3 cells and transiently transfected COS-7

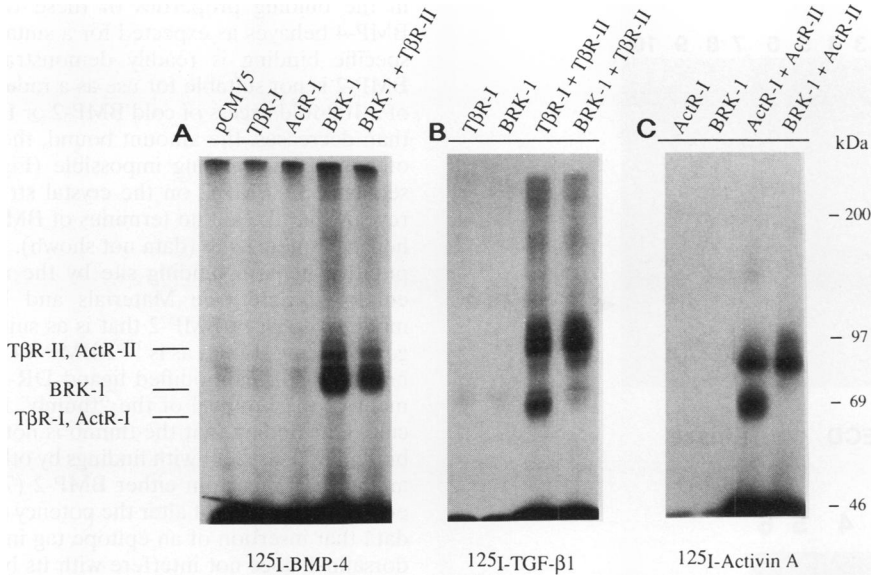

FIG. 6. TGF-β does not bind to BRK-1. COS-1 cells transfected with the indicated cDNAs in the pCMV5 or pJT4 vector or transfected with pCMV5 alone were affinity labeled by incubation with 250 pM ^{125}I-BMP-4, ^{125}I-TGF-β_1, or ^{125}I-activin A as indicated. Cell lysates were subjected to SDS-PAGE and autoradiography. The positions of the affinity-labeled products and the molecular mass markers are indicated.

cells (Fig. 7A and B) suggests that the BMP type I receptors present in NIH 3T3 cells are BRK-1 or a closely related isoform. Furthermore, BRK-1, or a closely related isoform, appears to associate with additional BMP-labeled proteins in NIH 3T3 cells.

To further compare the binding properties of BRK-1 with that of the endogenous receptor in NIH 3T3 cells, we performed competition assays comparing BMP-4 binding affinities in NIH 3T3 cells and in COS-7 cells in which BRK-1 was transiently overexpressed (Fig. 7C). The differences in the IC_{50} for BMP-4 in NIH 3T3 cells in this experiment (Fig. 7C) and those reported in Table 1 can be explained by the fact that the tracer concentrations used in this experiment were higher than those in the experiment summarized in Table 1. Interestingly, the affinity of unlabeled BMP-4 for BRK-1 transiently overexpressed in COS-7 cells was approximately 10-fold lower than that observed in NIH 3T3 cells (IC_{50}, 1450 pM for BRK-1 in COS-7 cells versus 172 pM in NIH 3T3 cells [Fig. 7C]). The complex formation detected between native BRK-1 and additional proteins in NIH 3T3 cells (Fig. 7B) suggests that the difference in affinity observed for BMP-4 to BRK-1 in COS-7 cells versus that observed for BMP-4 in NIH 3T3 cells might be due to the formation of a higher-affinity complex between BRK-1 and a type II receptor in NIH 3T3 cells which is not expressed at sufficient levels in COS-7 cells.

BRK-1 associates with the BMP type II receptor DAF-4. In order to directly test the ability of BRK-1 to interact with a BMP type II receptor, we cotransfected it with *daf-4* (24) in COS-7 cells. When COS cells were cotransfected with the *BRK-1* and *daf-4* cDNAs, affinity-labeled DAF-4 protein coprecipitated with BRK-1 when either of the BRK-1 antibodies was used (Fig. 8). DAF-4 cross-linked to ^{125}I-BMP-4 migrates as a poorly labeled complex of 100 to 140 kDa which is larger than that of other type II receptors, primarily because of an extended carboxy terminus unique to DAF-4 (24). Conversely, affinity-labeled BRK-1 also coprecipitated with DAF-4 when *BRK-1* was cotransfected with a lower-molecular-weight version of *daf-4* in which a portion of the long C-terminal tail was replaced with the influenza virus hemagglutinin (HA) epitope (24) (Fig. 8). This indicates that BRK-1 is capable of forming a complex with a known BMP type II receptor in the presence of BMP-4.

Saturation binding assays (Table 2) indicated that the binding affinity of ^{125}I-BMP-4 in COS-7 cells transfected with *BRK-1* alone was not significantly different from the affinity in cells transfected with both *BRK-1* and *daf-4*. The affinity of ^{125}I-BMP-4 to the native receptor in NIH 3T3 cells (K_d, ~250 pM [Fig. 2]) was closest to that observed when both BRK-1 and DAF-4 were present together in COS-7 cells (K_d, ~343 pM [Table 2]). A more dramatic difference in affinity to BRK-1 compared with the receptor in NIH 3T3 cells was observed for the unlabeled BMP-4 (Fig. 7C) than was observed in saturation assays with iodinated BMP-4 (Table 2 versus Fig. 2), suggesting that iodination interferes with complex formation of the type I and type II receptors. Additional work will be needed to determine whether unlabeled BMP-2 and BMP-4 exhibit a differential affinity to the different receptor forms and whether significant changes in binding affinity are achieved during complex formation of the type I and type II receptors.

Tissue distribution of BRK-1 mRNA. With a *BRK-1* extracellular domain probe, two major *BRK-1* mRNA species of ~6.2 and 3.6 kb were detected in various mouse tissues by Northern analysis (Fig. 9). The tissue distribution of *BRK-1* mRNA is quite widespread, with the highest levels present in the heart and brain. The 6.2-kb transcript was more abundant than the 3.6-kb transcript in all tissues examined. Similar results were obtained with a kinase domain probe or a full-length cDNA probe (data not shown).

DISCUSSION

BMP-2 and BMP-4 share the same receptors in NIH 3T3 cells. We have demonstrated that BMP-2 and BMP-4 interact with the same binding proteins in NIH 3T3 cells and that these binding proteins resemble those which would be expected for the type I and type II receptors of the TGF-β superfamily. BMP-2 and BMP-4 are able to compete for binding to the same set of binding proteins. Nevertheless, a notable difference

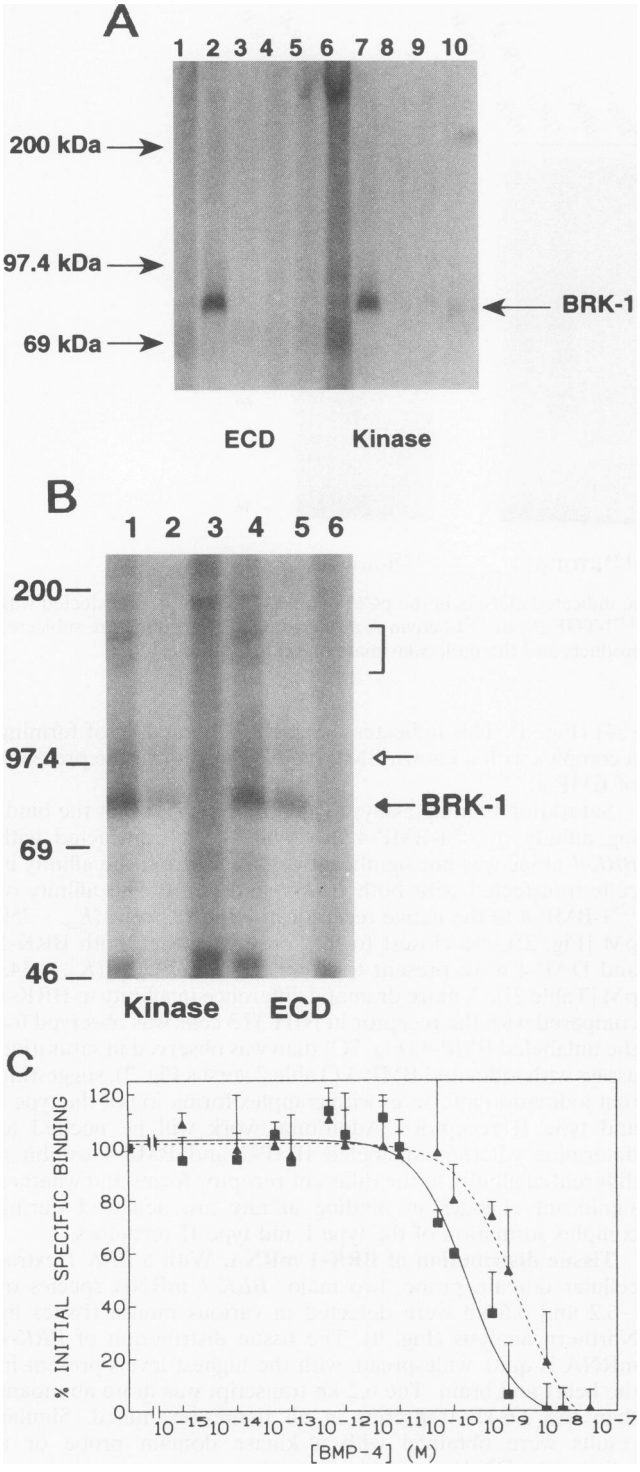

FIG. 7. (A) Immunoprecipitation of affinity-labeled BMP–BRK-1 complexes with either ECD or kinase domain (Kinase) polyclonal antibodies. Confluent 10-cm-diameter plates of COS-7 cells transiently transfected with *BRK-1* were affinity labeled with 141 pM ^{125}I-BMP-4 alone (lanes 1, 2, 6, and 7) or in the presence of 30 nM DR-BMP-2 (lanes 3 and 8), 30 nM BMP-2 (lanes 4 and 9), or 30 nM BMP-4 (lanes 5 and 10). After cross-linking with 0.135 mM DSS, cells were extracted and radioimmunoprecipitated with the ECD antibody (lanes 2 to 5) or preimmune serum (lane 1) or the kinase antibody (lanes 7 to 10) or preimmune serum (lane 6). Immunoprecipitated proteins were separated by SDS-PAGE on 7.5% polyacrylamide gels. The positions of affinity-labeled BRK-1 and molecular mass markers are indicated. (B) Immunoprecipitation of affinity-labeled BMP–BRK-1 complexes from NIH 3T3 cells. NIH 3T3 cells were grown to confluence on 10-cm-diameter plates and affinity labeled with 321 pM ^{125}I-BMP-4. After cross-linking with 0.135 mM DSS, cells were extracted and radioimmunoprecipitated with antisera generated against either the kinase domain (lanes 1 and 2) or the ECD (lanes 4 and 5) of BRK-1 or preimmune serum (lanes 3 and 6). The samples in lanes 2 and 5 were boiled in 1% SDS for 1 min prior to immunoprecipitation. Immunoprecipitated proteins were separated by SDS-PAGE on 7.5% polyacrylamide gels and visualized by autoradiography after a 3-week exposure with intensifying screens. The experiment was repeated once with similar results. The positions of affinity-labeled proteins and molecular mass markers are indicated. (C) Comparison of BMP-4 binding affinity in NIH 3T3 cells versus COS-7 cells transiently transfected with BRK-1. Confluent 12-well plates were affinity labeled with 267 pM ^{125}I-BMP-4 in the presence of increasing concentrations of unlabeled BMP-4. The IC_{50}s generated by INPLOT were 172 pM for NIH 3T3 cells (squares) and 1,450 pM for BRK-1 transfected COS-7 cells (triangles). Similar results were obtained in two additional experiments.

in the binding properties of these two ligands exists. ^{125}I-BMP-4 behaves as expected for a suitable radioligand, in that specific binding is readily demonstrable. In contrast, ^{125}I-BMP-2 is not suitable for use as a radioligand, as the addition of a 100-fold excess of cold BMP-2 or BMP-4 increases, rather than decreases, the amount bound, thereby making estimates of nonspecific binding impossible (Fig. 1). Modeling of the sequence of BMP-2 on the crystal structure of TGF-β_2 (19) reveals that the amino terminus of BMP-2 contains a putative heparin binding site (data not shown). Indeed, removal of this putative heparin binding site by the mild trypsin conditions employed here (see Materials and Methods) results in a modified form of BMP-2 that is as suitable for use in radioligand binding studies as is ^{125}I-BMP-4 (Fig. 1). Accordingly, we have named this modified ligand DR-BMP-2 in acknowledgment of the removal of the "thumb" from the BMP-2 molecule. Our finding that the thumb is not necessary for receptor binding is consistent with findings by others that removal of the amino terminus from either BMP-2 (73) or OP-1 (53) either enhances or does not alter the potency of these ligands and the data that insertion of an epitope tag in the amino terminus of dorsalin-1 does not interfere with its biological activity (5). It remains to be seen whether this partial removal of the amino terminus yields a ligand that behaves as a full agonist, partial agonist, or antagonist in each of the biological assays associated with these molecules.

^{125}I-BMP-4 binds to a single population of binding sites with a K_d of ~250 pM (Fig. 2). The low site number (~1,400 sites per cell) precluded the use of higher concentrations of cold ligand because of the unacceptably high nonspecific binding observed at concentrations of >1 nM, making it impossible to exclude the possibility that multiple affinity states, and/or binding to multiple proteins with differential affinities, occur with these radioligands. Indeed, binding of ^{125}I-BMP-4 to collagen with a subnanomolar affinity in PC-12 cells has been demonstrated (49). However, since BMP-4 and TGF-β bind to collagen with similar affinities (48, 49) and since TGF-β did not compete for BMP binding at either the whole-cell level (data not shown) or in affinity-labeling studies (Fig. 3), it seems likely that the high-affinity binding sites measured here (Fig. 2) represent binding to receptor proteins on the cell surface.

Competition and cross-competition studies with ^{125}I-BMP-4 and ^{125}I-DR-BMP-2 indicate that BMP-4 and BMP-2 interact with the same binding proteins at the whole-cell level, as

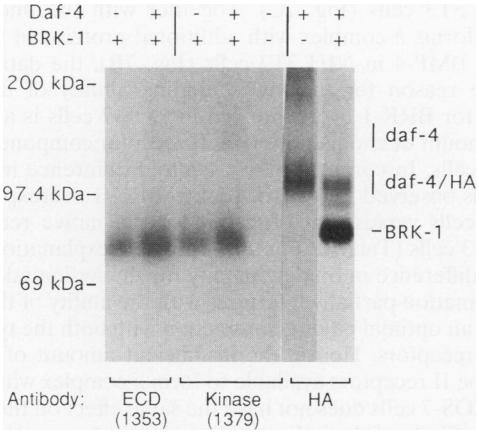

FIG. 8. Association of BRK-1 and DAF-4. ^{125}I-BMP-4 (200 pM) was bound and cross-linked to COS-7 cells transfected with 10 μg of *BRK-1* alone, in combination with 20 μg of *daf-4*, or with vector containing no cDNA insert (−). ^{125}I-BMP-4 (250 pM) was bound and cross-linked to COS-1 cells transfected with 8 μg of *daf-4*–HA alone or in combination with 8 μg of *BRK-1*. Cross-linked cells were extracted and radioimmunoprecipitated with an antibody raised against the ECD of BRK-1, an antibody raised against an intracellular kinase domain of BRK-1 (Kinase), or an antibody to the influenza virus HA epitope. Immunoprecipitated proteins were separated by SDS-PAGE and visualized by autoradiography. Positions of affinity-labeled proteins and molecular mass markers are indicated.

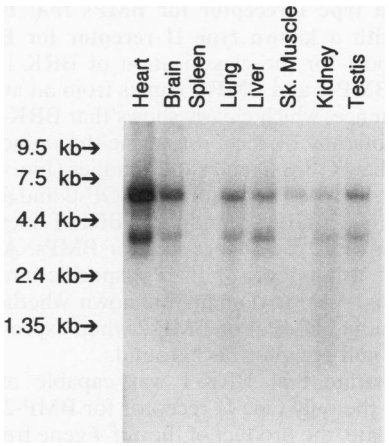

FIG. 9. Northern blot analysis. A mouse multiple tissue blot was probed with the extracellular domain of *BRK-1* cDNA. Each lane represents 2 μg of purified poly(A) RNA from the various mouse tissues listed on the upper margin of the blot. RNA size markers are indicated to the left of the blot.

complete displacement is obtained in each set of cross-competition experiments (data not shown). Furthermore, the K_d value for ^{125}I-BMP-4 (Fig. 2) and the IC_{50} values for the unlabeled ligands (Table 1), which assume a one-binding-site model, are consistent with the values obtained for these ligands in binding and functional assays in a variety of cell types (10, 18, 36, 47, 49).

Additional evidence that BMP-2 and BMP-4 interact with the same receptor complex comes from the affinity-labeling studies (Fig. 3). ^{125}I-DR-BMP-2 and ^{125}I-BMP-4 label distinct proteins of comparable size that correspond to the sizes expected for the type I and type II receptor proteins that have been characterized for TGF-β and activin (38). Notably, BMP-2, DR-BMP-2, and BMP-4 are able to compete for binding at the proteins labeled by either tracer, whereas TGF-β does not. Together, these data indicate that BMP-2 and BMP-4 interact with the same receptor proteins and that the BMP receptor complex is not accessible to either TGF-β_1 or TGF-β_2.

BRK-1 is a mammalian BMP receptor. The ligand-binding properties of the native receptor from NIH 3T3 cells are shared by the novel receptor, BRK-1, that was isolated from NIH 3T3 cells by a PCR cloning approach. The affinity of ^{125}I-BMP-4 for BRK-1 when transiently expressed in COS-7 cells is comparable to that obtained for the endogenous receptor in NIH 3T3 cells (K_d = ~497 versus ~250 pM in NIH 3T3 cells). After subtraction of the molecular mass of the cross-linked ligand monomer, the size of the protein believed to correspond to the type I receptor from NIH 3T3 cells is ~57 to 62 kDa (Fig. 3), and the size of the major band observed in *BRK-1*-transfected COS cells is ~64 kDa (Fig. 5B). Furthermore, the mass of the immunoprecipitated protein recognized by BRK-1 receptor-specific polyclonal antibodies in either COS cells transiently transfected with *BRK-1* or in native NIH 3T3 cells is ~62 to 71 kDa (after subtraction of the molecular mass of the ligand monomer) (Fig. 7A & B). This suggests that BRK-1, or a closely related isoform, is a component of the type I receptor band detected in the cross-linking assay in NIH 3T3 cells (Fig. 3).

Like the native receptor (Fig. 3), BRK-1 does not bind TGF-β, either by itself or in conjunction with the type II receptor (Fig. 6). Furthermore, BRK-1 does not bind activin, either by itself or in conjunction with the activin type II receptor (Fig. 6). Hence, the recent demonstration that activin can compete for ^{125}I-BMP-4 binding to a limited extent under certain circumstances (36) cannot be explained by an interaction at BRK-1 as a common receptor subunit. Moreover, the observation that both TGF-β and BMP-2 or BMP-4 can affect bone formation and remodeling (13, 72) cannot be explained by an interaction with BRK-1 as a common receptor subunit, as is seen for other cytokine receptor systems (54). It remains to be seen whether other type I receptors for either BMP-2 or BMP-4 or additional members of the BMP family are shared by TGF-β or activin.

Our Northern analysis suggests that this novel BMP receptor is present in a wide variety of tissues, although our binding data on a variety of cell lineages (data not shown) suggest that this is a low-level ubiquitous distribution. Surprisingly, the human homolog of BRK-1, ALK-3, which was recently described as an orphan receptor, appears to have a more restricted pattern of distribution in human tissues (60). The ubiquitous tissue distribution observed for the mouse receptor reported here is consistent with the broad range of activities reported for the BMPs in rodent tissues and cell lines (5, 10, 47, 49, 73).

TABLE 2. Comparison of the ^{125}I-BMP-4 binding affinities in COS-7 cells transiently transfected with different receptors[a]

Receptor cDNA	K_A ± SEM[b] (M [10^9])	K_d (M [10^{-10}])
BRK-1 alone	2.01 ± 0.53	4.97
daf-4 alone	1.17 ± 0.45	8.58
BRK-1 + *daf-4*	2.91 ± 0.55	3.43

[a] COS-7 cells were transfected with either 10 (*BRK-1*) or 20 (*daf-4*) μg of DNA, as described in Materials and Methods.

[b] In this case, SEM represents the approximate standard error in the parameter estimate.

BRK-1 is a type I receptor for BMPs that is capable of interacting with a known type II receptor for BMPs. More rigorous support for the classification of BRK-1 as a type I receptor for BMP-2 and BMP-4 comes from an analysis of the receptor sequence, which clearly shows that BRK-1 belongs in the type I subfamily of receptor serine/threonine kinases for members of the TGF-β ligand superfamilies (Fig. 4). However, in contrast to the type I receptors for TGF-β and activin (1, 11, 22, 23, 25, 57, 66, 76), BMP binding to BRK-1 does not require cotransfection of a type II receptor for BMPs. As we consistently observe a low level of BMP-4-specific binding sites in COS cells (Fig. 5A), it remains unknown whether BRK-1 is capable of binding BMP-4 or BMP-2 when expressed alone in a completely null receptor background.

To demonstrate that BRK-1 was capable of forming a complex with the only type II receptor for BMP-2 and BMP-4 identified to date, the product of the *daf-4* gene from *C. elegans* (24), polyclonal antibodies were generated against a region of the BRK-1 kinase domain that had previously been demonstrated to yield isoform-specific antibodies (33). Analysis of the cAMP-PK (78) and CDK2 (20) crystal structures indicates that this region is highly exposed on the surface of the molecule and apparently quite antigenic. Comparison of the protein sequences from this region in all members of the receptor serine/threonine kinase family cloned to date indicates that BRK-1, ALK-6, and RPK1, which correspond to the same or closely related receptors from mice (ALK-6) and chickens (RPK1), should be the only known kinases recognized by this antibody (Fig. 4A and data not shown). The specificity of the BRK-1 kinase domain antibody is further confirmed by its ability to immunoprecipitate proteins with sizes similar to those of proteins immunoprecipitated by antibodies against the BRK-1 extracellular domain (Fig. 7A and B).

Immunoprecipitation with either the extracellular domain antibody to BRK-1 or the antipeptide kinase domain antibody clearly demonstrates that BRK-1 is capable of forming a complex with DAF-4 in the presence of BMP-4 (Fig. 8). This complex formation is also demonstrated when BRK-1 is immunoprecipitated with an HA epitope-tagged version of DAF-4, using an antibody to HA (Fig. 8). In this respect, BRK-1 behaves in a fashion similar to the cloned type I receptors for activin and TGF-β (1, 11, 22, 25, 57, 66, 76), in that the receptor is capable of forming a complex with the type II receptor. It is possible that this interaction is nothing more than a protein-protein interaction of the two receptor subunits, driven by the overexpression of the receptors in the COS cell system. Nevertheless, the change in labeling intensity of the BRK-1 band in the presence of DAF-4 (Fig. 8) implies that a conformational change that potentially alters the efficiency of cross-linking of ^{125}I-BMP-4 to BRK-1 in the receptor complex has taken place. This observation suggests that the BMP-4 ligand might interact differently with BRK-1 when the type I receptor is present in a complex with the type II receptor. Whether or not DAF-4 and BRK-1 form a preexisting complex on the cell surface and whether or not the complex of DAF-4 and BRK-1 is capable of producing a signal in response to BMP-2 or BMP-4 remain to be determined.

At the present time, it is not known whether formation of a complex between the type I and type II receptors will result in formation of a higher-affinity state of the receptor for BMP-4. In this respect, it is interesting to compare the properties of binding of both iodinated and unlabeled BMP-4 to BRK-1 expressed in COS-7 cells with those of native receptors present in NIH 3T3 cells. The affinity of unlabeled BMP-4 in COS-7 cells transfected with BRK-1 alone is approximately an order of magnitude lower than that observed for the native receptors in NIH 3T3 cells (Fig. 7C). Together with the finding that BRK-1 forms a complex with additional proteins in the presence of BMP-4 in NIH 3T3 cells (Fig. 7B), the data suggest that the reason for the lower binding affinity of unlabeled BMP-4 for BRK-1 overexpressed in COS-7 cells is an insufficient amount of endogenous type II receptor component in the COS-7 cells. In contrast, only a twofold difference in binding affinity is observed for ^{125}I-BMP-4 to BRK-1 overexpressed in COS-7 cells versus that observed for the native receptor in NIH 3T3 cells (Table 2; Fig. 2). A possible explanation for the lack of difference in binding affinity for the iodinated ligand is that iodination partially interferes with the ability of the ligand to form an optimal binding interaction with both the type I and type II receptors. Hence, the insufficient amount of endogenous type II receptors available to form a complex with BRK-1 in the COS-7 cells does not have the same effect on the relative binding affinity of the iodinated ligand as it does on the affinity of the unlabeled ligand. Consistent with this observation, cotransfection of the BMP type II receptor DAF-4 with BRK-1 does not result in an affinity for ^{125}I-BMP-4 that is substantially different from that seen in cells transfected with BRK-1 alone, although a slight increase is observed over that seen in cells transfected with DAF-4 alone (Table 2).

The sequences of the type I receptor and the type II receptor subfamilies suggest that they might possess differences in substrate specificity. The TREEANALYSIS program reveals that the type I receptor subfamily, which includes BRK-1, RPK1 (56), ALK-6 (61), ALK-3 (60), ActRI (SKR-1, TSK7L, R1, and ALK-2) (1, 23, 31, 41, 60), ActRIB (SKR-2, R2, and ALK-4) (11, 31, 60, 77), TSR1 (R3 and ALK-1) (1, 31, 60), and ALK-5 (R4) (25, 31), bears the highest similarity to Cek, Elk, Hek, Mek, Eck, Eph, and Nuk receptor tyrosine kinase (52), Abl and DASH tyrosine kinase (8), Raf serine/threonine kinase (9, 46), and Csk tyrosine kinase (8) families (63, 62, 62, and 62% similarity in kinase domains I to XI, respectively). In contrast, the TGF-β (35, 65) and activin (2, 14, 40) type II receptor subfamily, the C14 putative MIS type II receptor (3), and the DAF-4 type II receptor for BMPs (24) do not exhibit a similarity score above 60% to kinases other than putative receptor serine/threonine or cytosolic serine/threonine kinases. This difference in relative similarity to kinase families with different substrate specificities may indicate differences in the preferential substrate specificity of the type I and type II receptors for the TGF-β family members and provides an explanation for the observation that both receptor types are needed for TGF-β (6, 11, 25, 75) and activin (1, 11) signaling. The degree of sequence homology of BRK-1 to the type I receptors for TGF-β (1, 6, 23, 25, 57) and activin (1, 11, 22, 66, 76), coupled with the demonstrated ability to bind BMP-2 and BMP-4, allows the classification of BRK-1 as a type I receptor for BMP-2 and BMP-4. Just as the TGF-β and activin type I receptors form a complex with their corresponding type II receptors in the presence of ligand (1, 11, 22, 25, 32, 57, 66, 75, 76), BRK-1 also forms a complex with the DAF-4 BMP type II receptor. Hence, it is tempting to speculate that BMP receptor signaling also requires the presence of both the type I and type II receptors. However, the possibility that BMP-2 and BMP-4 can signal solely through the BRK-1 receptor cannot be excluded at the present time.

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