Identification of a Human Type II Receptor for Bone Morphogenetic Protein-4 That Forms Differential Heteromeric Complexes with Bone Morphogenetic Protein Type I Receptors*

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Bone morphogenetic proteins (BMPs) comprise the largest subfamily of TGF-β-related ligands and are known to bind to type I and type II receptor serine/threonine kinases. Although several mammalian BMP type I receptors have been identified, the mammalian BMP type II receptors have remained elusive. We have isolated a cDNA encoding a novel transmembrane serine/threonine kinase from human skin fibroblasts which we demonstrate here to be a type II receptor that binds BMP-4. This receptor (BRK-3) is distantly related to other known type II receptors and is distinguished from them by an extremely long carboxy-terminal sequence following the intracellular kinase domain. The BRK-3 gene is widely expressed in a variety of adult tissues. When expressed alone in COS cells, BRK-3 specifically binds BMP-4, but cross-linking of BMP-4 to BRK-3 is undetectable in the absence of either the BRK-1 or BRK-2 BMP type I receptors. Cotransfection of BRK-2 with BRK-3 greatly enhanced affinity labeling of BMP-4 to the type I receptor, in contrast to the affinity labeling pattern observed with the BRK-1 + BRK-3 heteromeric complex. Furthermore, a subpopulation of super-high affinity binding sites is formed in COS cells upon cotransfection only of BRK-2 + BRK-3, suggesting that the different heteromeric BMP receptor complexes have different signaling potential.

Bone morphogenetic proteins (BMPs) are the largest subfamily of growth factors in the TGF-β superfamily and have been demonstrated to play important roles in endochondral bone formation and embryogenesis (1–4). Like other members of the TGF-β superfamily, BMPs appear to interact with type I and type II receptors on the cell surface (5). Following the expression of the type II receptors for activin and TGF-β (6, 7), an increasing number of transmembrane serine/threonine kinases have been identified in mammals based on the conserved amino acid sequences in the intracellular domain (8, 9). These include three distinct mammalian type I receptors for BMPs that are distinguished from the type I receptors for TGF-β (10–12) and activin (11, 13) by the capability of binding ligand on their own when transfected into COS cells (5, 14, 15). BRK-1 (also known as ALK-3, TFR11, and BMPR-IA) binds BMP-2 and BMP-4 more efficiently than it binds BMP-7 (5, 14, 15). BRK-2 (also known as ALK-6, BMPR-IB, and RPK-1 (16)) binds both BMP-4 and BMP-7 efficiently (15). ActRI (also known as ALK-2 and SKR1) binds both activin and BMP-7 but does not bind BMP-4 (15, 17).

The only type II receptors that have been identified for BMPs to date are from non-mammalian sources. The product of the daf-4 gene from Caenorhabditis elegans binds both BMP-2 and BMP-4 (18) and forms a complex with each of the BMP type I receptors in the presence of ligand (5, 15). The product of the pune gene from Drosophila, originally identified as the Drosophila homologue of the activin type II receptor (AtrII (19)), has recently been demonstrated to bind BMP-2 and is required for signaling by the Drosophila homologue of BMP-2 and BMP-4, the product of the decapentaplegic gene (20, 21). The ability of the mammalian BMP type I receptors to form a complex with the nematode Daf-4 type II receptor as well as with other proteins having the expected size of the type II receptor in endogenous systems (5, 15) suggests the existence of mammalian BMP type II receptors. Indeed, expression of a BMP type I receptor in COS cells is insufficient to reproduce the high affinity binding observed in endogenous systems, suggesting that the high affinity complex is composed of both type I and type II receptor subunits (5). Since complex formation between the type I and type II receptors is a prerequisite for signal transduction induced by TGF-β-related ligands (13, 22), identification of the mammalian BMP type II receptor(s) is necessary in order to fully understand the nature of the BMP receptor signaling complex.

In this paper, we describe the cloning and characterization of a human BMP type II receptor, which we term BRK-3 (BMP Receptor Kinase-3) that is identical in sequence to a novel receptor Ser/Thr kinase isolated on the basis of its ability to interact with type I receptor kinase domains in the yeast two hybrid system (23). As is observed for the activin and TGF-β type II receptors (6, 7), BRK-3 binds ligands on its own when expressed in COS cells. However, in contrast to what is established for other type II receptors, cross-linking of 125I-labeled BMP-4 to this human BMP type II receptor is not observed in the absence of a BMP type I receptor. We demonstrate that, while BRK-3 is capable of forming a complex with either the BRK-1 or BRK-2 BMP type I receptors, a high affinity complex

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1 The abbreviations used are: BMP, bone morphogenetic protein; TGF-β, transforming growth factor-β; BMP, BMP receptor kinase; PCR, polymerase chain reaction; tBRK-3, truncated form of BRK-3; MISRII, Mullerian-inhibiting substance receptor type II.
is formed only when BRK-2 is coexpressed with BRK-3 in COS cells.

EXPERIMENTAL PROCEDURES

Cloning of the Human BRK-3 Receptor—PCR was carried out using approximately 0.2-μg cDNAs prepared from human skin fibroblasts mRNA as a template and the following degenerate primers: TK1 (sense, kinase subdomain II), 5′-GAAGCTGCGTNNAGAAG(TG)NNTTT-3′; AYR5 (sense, kinase subdomain IV), 5′-ATGAAGAAGAATGCTCTTGG-3′; TK4 (anti-sense, kinase subdomain VIIB), 5′-CAAGGTAGCAAGTTTAGNTNAAG(TG)NNTTT-3′; and TSK2 (anti-sense, kinase subdomain VIII), 5′-GACTCTCTCGNNGCNGTA(G)-3′ (Promega).

cDNA libraries of human skin fibroblasts were constructed in the λgt10 vector using SuperScript Choice System (Life Technologies, Inc.), yielding a 2 × 107 independent recombinants. Hybridization screening with one of the PCR clones was done as described (24). Nucleotide sequence determination was performed on both strands using an ABI DNA sequence 373 after subcloning several restriction fragments into suitable vectors such as M13mp18mp19.

Expression of BMP Receptors in COS Cells—The binding studies described here were performed after expression of a truncated form of the full-length hBRK-3 in COS cells because of initial difficulties in obtaining the full-length hBRK-3 clone due to the extremely long carboxyl terminus. To construct the truncated form of BRK-3 (tBRK-3), a stop codon was inserted after Ile540 in the carboxyl terminus with the aid of PCR, generating a truncated carboxyl terminus corresponding to the length of the activin type I receptor (6). The stop codon was inserted with the primer, 3′-CGCCTACAAATTCTTGGACAC-5′ (sense, kinase subdomain VIII), 5′-GACTCTCTCGNNGCNGTA(G)-3′, corresponding to the antisense sequence of nucleotides 2013–2137 after subcloning several restriction fragments into suitable vectors such as M13mp18mp19.

RESULTS

To isolate a novel human receptor protein kinase related to type II receptors for TGF-β and activin, we first employed PCR to amplify cDNA from human skin fibroblasts. In addition to the human activin type II receptor cDNA (25), we obtained a 289-nucleotide PCR fragment encoding a portion of a novel Ser/Thr kinase. We isolated several overlapping cDNAs from human fibroblast libraries by hybridization screening. Analysis of the entire coding sequence reveals the BRK-3 protein to be a 1038-amino acid member of the transmembrane Ser/Thr kinase family, identical to the T-ALK receptor recently identified from a HeLa cell cDNA library (23), although we have isolated additional 5′- and 3′-noncoding regions of the cDNA. The sequence of BRK-3 has been deposited to the GSDDB/DDJ/EMBL/NCBI data bases under the accession number D50516.

Several structural features suggest that the BRK-3 protein is a type II receptor for a member of the TGF-β superfamily. First, both an upstream Cys box in the extracellular domain and an intracellular GS domain that are conserved only in type I receptor sequences (5, 9, 22) are absent in the BRK-3 sequence. Second, the sequence of the BRK-3 kinase domain is more closely related to other type II receptors, particularly the MSLII (26, 27), activin type II (6) and IIB (28) receptors, than it is to the type I receptors (data not shown and (23)). Third, BRK-3 has a unique 533-amino acid carboxyl-terminal tail rich in Ser, Thr, and Pro residues that is absent in the type I receptors.

Fig. 1 shows the tissue specific expression of the BRK-3 gene as determined by Northern blot analysis in different human tissues. Four distinct transcripts of >10, 8, 6.5, and 5 kilobase pairs were detected in almost all tissues examined, except liver, leukocyte, testis, and thymus, where the longest transcript of the BRK-3 gene was not detected. The BRK-3 gene was ex-
pressed abundantly in lung, placenta, and testis. The relative abundance of the three major transcripts (>10, 8, and 5 kilobase pairs) varied depending on the tissue and may reflect tissue-specific processing of the transcripts.

To determine whether the BRK-3 protein binds BMP-4, COS-7 cells were transfected with tBRK-3 alone or in combination with either the BRK-1 or BRK-2 type I receptor, and whole cell binding to a single concentration of 125I-labeled BMP-4 was measured (Fig. 2). tBRK-3 binds 125I-labeled BMP-4 by itself when expressed alone in COS cells. Binding of 125I-labeled BMP-4 is greatly enhanced by coexpression of tBRK-3 with BRK-2 and to a lesser extent by coexpression of tBRK-3 with BRK-1. These increases in binding could be due either to an increase in binding affinity or to an additive effect of binding to the different receptor proteins. In contrast to the whole cell binding experiments, affinity labeling experiments reveal that tBRK-3 is not efficiently cross-linked by 125I-labeled BMP-4 when expressed alone in COS-1 cells (Fig. 3A). However, coexpression of tBRK-3 with either BRK-1 or BRK-2 reveals a new band at ~94 kDa, in addition to the cross-linked type I receptor bands at ~75–78 kDa. (All approximate molecular weights are assumed to include the molecular weight of the BMP-4 monomer.) Allowing for the presence of three potential N-glycosylation sites in the BRK-3 extracellular domain, the new ~94-kDa band is consistent with the predicted molecular weight of tBRK-3, cross-linked to the BMP-4 monomer. Interestingly, coexpression of BRK-2 with BRK-3 results in a more intense labeling of the type I receptor band, in contrast to what is observed when the BRK-1-type I receptor is coexpressed with BRK-3. The ~150-kDa band that is most apparent in the BRK-2 + BRK-3 cotransfected cells may represent cross-linking with endogenous BRK-3 (see below). The nature of the minor ~110-kDa band that is observed when BRK-3 is coexpressed with either type I receptor is unknown and could be due either to differential glycosylation or cross-linking of the ligand dimer to tBRK-3.

Immunoprecipitation of cells cotransfected with tBRK-3 and either BRK-1 or BRK-2 resulted in the appearance of the same additional bands apparent in the cross-linking studies, indicating that tBRK-3 forms a complex with either type I receptor in the presence of 125I-labeled BMP-4 (Fig. 3B). Furthermore, an additional band of ~150–180 kDa is apparent in the BRK-2 receptor immunoprecipitates (Fig. 3B), suggesting that an additional protein exists in the complex of BRK-2 with tBRK-3 in the presence of 125I-labeled BMP-4. Interestingly, this additional protein corresponds in size to the full-length BRK-3 receptor cross-linked to the BMP-4 monomer, and may represent the endogenous COS cell BRK-3 that exists in the complex with the truncated type II receptor in the presence of ligand and the type I receptor. When affinity labeling to the BRK-2 + BRK-3 receptor complex was performed with 125I-labeled BMP-4 in the absence and presence of 10 nM BMP-2, DR-BMP-2 (5), or BMP-4, binding was completely abolished to all bands in the BRK-2 + BRK-3 complex, whereas 50 nM TGF-β1 was completely ineffective, indicating that this receptor complex specifically binds either BMP-2 or BMP-4, but is not accessible to TGF-β1 (data not shown). Finally, the differential change in affinity labeling of the BRK-1 versus BRK-2 band that is observed in the type I-BRK-3 receptor complex is in contrast to what is observed when DAF-4 is cotransfected with either type I receptor, where an increase in affinity labeling of either type I receptor is observed (Fig. 3B). These results suggest that, whereas similar increases in either binding affinity or affinity labeling occur when DAF-4 is the type II receptor, tBRK-3 forms different complexes with the two different type I receptors.

In order to address the possibility that the differential affinity labeling of the BRK-1 or BRK-2 receptor bands that is observed in the presence of BRK-3 is reflective of a differential nature of the receptor complex that is dictated by the type I receptor, the binding affinity of BMP-4 to the two different receptor complexes was examined. Fig. 4A compares the affinity of BMP-4 in cells transfected with either BRK-1 alone to that in cells transfected with BRK-1 + BRK-3. No change in binding affinity for BMP-4 is observed (IC50 = 1.24 × 10−9 M for BRK-1 alone versus 1.25 × 10−9 M in cells cotransfected with BRK-1 + BRK-3). Although the binding affinity of BMP-4 is similar in cells transfected with either BRK-1 or BRK-2 alone, cotransfection of BRK-3 with BRK-2 results in an increase in the binding affinity of BMP-4 (IC50 = 4.58 × 10−9 M for BRK-2 alone versus 3.35 × 10−12 M and 1.34 × 10−8 M in cells cotransfected with BRK-2 + BRK-3 (Fig. 4B)). Further-

2 T. Nohno, T. Ishikawa, T. Saito, K. Hosokawa, S. Noji, J. Ting, B. B. Koenig, A. L. Pequet, and J. S. Rosenbaum, unpublished observations.
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Fig. 4. BMP-4 binds to the different receptor complexes with differential affinity. A, comparison of BMP-4 binding affinity in COS-7 cells transfected with BRK-1 alone or with BRK-1 and BRK-3. Whole cell binding was performed with 107 pM 125I-labeled BMP-4. The points represent the average of triplicate determinations. Error bars represent the standard error of the mean. The curves represent the optimal fit of the data to a one binding site model. The IC50 values are reported in the text. B, comparison of BMP-4 binding affinity in COS-7 cells transfected with BRK-2 alone or with BRK-2 and BRK-3. Whole cell binding was performed with 150 pM 125I-labeled BMP-4. The points represent the average of triplicate determinations. Error bars represent the standard error of the mean. The curves represent the optimal fits of the data, to a one binding site model (BRK-2) or to a two binding site model (BRK-2 + BRK-3). The two binding site model assumes that the tracer binds to both sites with similar affinity, but the unlabeled ligand distinguishes between the two affinity sites.

more, the formation of a super-high affinity binding site population (where the data are best described by a two binding site model for the competitor, p < 0.0001) is observed only in cells cotransfected with the BRK-2 type I receptor and the BRK-3 type II receptor.

DISCUSSION

We have isolated a new member of the receptor Ser/Thr protein kinase superfamily that is structurally related to the type II receptors for members of the TGF-β superfamily. When expressed in COS cells, this human type II receptor is capable of forming differential heterodimeric complexes with either the murine BRK-1 or the chicken BRK-2 type II receptors. In the presence of BMP-4, it has been demonstrated previously that murine and human BMP type I receptors form a complex with the nematode DAF-4 type II receptor (15), that a dominant negative construct of the murine BMP type I receptor BMPRIA/TFR11/BRK-1 alters dorsal-ventral patterning when expressed in ventral blastomeres of Xenopus embryos (14), that the chicken BMP-related ligand dorsalin-1 induces alkaline phosphatase activity in murine bone marrow stromal cells (29), and that the Drosophila BMP-2 and BMP-7 homologues, dpp and 60A, induce ectopic bone formation in rats (30). Given the high degree of sequence conservation among the ligands and receptors of this family, it is unlikely that the observed differential binding properties for the type I-type II receptor complexes described herein are due to the species differences of the type I receptors. Rather, the differential binding properties described below most likely reflect the different signaling potential of the receptor complexes, which has been demonstrated for the TGF-β and activin receptor systems to be dependent on the type I receptor in the complex (13).

The mammalian BRK-3 receptor binds BMP ligands on its own when transfected into COS cells, as is observed for mammalian type II receptors for TGF-β1 (7), activin (6, 28), M1S (27), and with the nematode BMP type II receptor DAF-4 (18). The finding that BRK-3 binds ligands on its own in COS cells, coupled with the finding that both truncated BRK-3 and a protein that corresponds in size to the full length BRK-3 can be brought down in a complex with the type I receptor when BRK-2 and truncated BRK-3 are cotransfected into COS cells (Fig. 3B) is consistent with the model that these receptors are heterotetramers, composed of pre-existing homodimers of the type II receptor (31–36). We cannot say at the present time whether the BRK-3 type II receptor homodimer is formed in the absence of ligand.

In contrast to other type II receptors, cross-linking to BRK-3 is not evident in the absence of the type I receptor (Fig. 3A). A sequential binding model for TGF-β and activin receptors has been proposed wherein the ligand first binds to a preexisting type II receptor homodimer, with subsequent association of the type I receptor into the complex (22, 31, 32, 35, 36). It has also been proposed that recruitment of the type I receptor into the complex causes a conformational change in either the ligand or the receptor such that the ability of the lysines on the ligand and the receptor to cross-link is altered, resulting in an increased cross-linking efficiency of the type I versus the type II receptors (37, 38). In contrast to the TGF-β and activin receptor systems, it appears that the conformational change in the BMP ligand-receptor complex that occurs when both the type I and type II receptors contact the ligand results in an increased tendency for the ligand to be cross-linked to the BMP type II receptor. Additionally, in contrast to the TGF-β and activin receptor systems, both BMP type I (5) and type II receptors are capable of binding BMP ligand on their own when transfected into COS cells. Hence, it cannot be said at the present time whether the same sequential binding model applies to the BMP receptor complex. Nevertheless, the distinction between the ability to bind and to cross-link to the receptor appears to be an important one for the BRK-3 mammalian BMP type II receptor, and it appears that a conformational change in either the ligand or the type II receptor is taking place as the type I receptor is recruited into the complex. While this does not seem to be the case for the nematode BMP type II receptor DAF-4, which does cross-link to BMPs in the absence of a cotransfected type I receptor (18), it is interesting that a similar result is obtained for the Drosophila Punt receptor, in which cross-linking to 125I-labeled activin is apparent in the absence of a cotransfected activin type I receptor (19), whereas cross-linking to 125I-labeled BMP-2 is not apparent in the absence of a cotransfected Drosophila BMP type I receptor (21), implying a differential distribution of lysines at the ligand-type II receptor interface for activin versus BMPs.

We also observe a dramatic difference in the binding affinity of BMP-4 to the different type I-BRK-3 type II receptor complexes (Fig. 4). While both the BRK-1 and BRK-2 type I receptors are capable of forming a complex with BRK-3, as evidenced by immunoprecipitation studies (Fig. 3B), an increase in binding affinity of BMP-4 is only observed when the BRK-2-BRK-3 complex is formed in COS cells. After submission of this manuscript, Liu et al. (39) reported the doing of an alternatively spliced human BMP type II receptor that is 10 amino acids shorter than the artificially truncated form of human BRK-3 studied here. Affinity labeling studies with 125I-labeled BMP-2 and 125I-labeled BMP-7 demonstrate complex formation of BRK-3 with ALK-3 (the human homologue of
BRK-1, ALK-6 (the murine homologue of BRK-2), and ActRI/ SKR1 in the presence of both ligands but the largest signal was observed when BMP-7 was the ligand and ActRI was the type I receptor (39). Interestingly, BMP-2 did not signal at all when BMP-1 or BRK-2 was the type I receptor. These authors did not report the binding affinity of these ligands to the different receptor complexes. The increase in binding affinity that we observe with BMP-4 in the presence of the appropriate receptor components is analogous to what is observed for other multi-component cytokine receptor systems, in which it is the high affinity state of the receptor that is responsible for signaling (40). We predict that the BRK-2-BRK-3 receptor complex will represent a functional signaling complex for low concentrations of BMP-4, whereas the BRK-1-BRK-3 complex will be insufficient to transduce the BMP-4 signal at low concentrations of ligand. It therefore follows that a requirement for formation of a functional receptor signaling complex for BMPs will not only be that the individual receptor subunits bind ligand and form a complex, but that they form a high affinity complex in the presence of the appropriate ligand. In addition, the signaling receptor subunits will need to be colocalized in the same cell or tissue in vivo in order to represent a physiologically relevant receptor complex. Investigations of the signaling potential of the different BMP receptor complexes, as well as the colocalization of the individual BMP receptor subunits in vivo are currently in progress.

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