The Mode of Bone Morphogenetic Protein (BMP) Receptor Oligomerization Determines Different BMP-2 Signaling Pathways*

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Bone morphogenetic proteins (BMPs) are multifunctional proteins regulating cell growth, differentiation, and apoptosis. BMP-2 signals via two types of receptors (BRI and BRII) that are expressed at the cell surface as homomeric as well as heteromeric complexes. Prior to ligand binding, a low but measurable level of BMP-receptors is found in preformed hetero-oligomeric complexes. The major fraction of the receptors is recruited into hetero-oligomeric complexes only after ligand addition. For this, BMP-2 binds first to the high affinity receptor BRI and then recruits BRII into the signaling complex. However, ligand binding to the preformed complex composed of BRII and BRI is still required for signaling, suggesting that it may mediate activating conformational changes. Using several approaches we have addressed the following questions: (i) Are preformed complexes incompetent of signaling in the absence of BMP-2? (ii) Which domains of the BRII receptors are essential for this complex formation? (iii) Are there differences in signals sent from BMP-induced versus preformed receptor complexes? By measuring the activation of Smads, of p38 MAPK and of alkaline phosphatase, we show that the ability of kinase-deficient BRII receptor mutants to inhibit BMP signaling depends on their ability to form heteromeric complexes with BRI. Importantly, a BRII mutant that is incapable in forming preassembled receptor complexes but recruits into a BMP-induced receptor complex does not interfere with the Smad pathway but does inhibit the induction of alkaline phosphatase as well as p38 phosphorylation. These results indicate that signals induced by binding of BMP-2 to preformed receptor complexes activate the Smad pathway, whereas BMP-2-induced recruitment of receptors activates a different, Smad-independent pathway resulting in the induction of alkaline phosphatase activity via p38 MAPK.

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complexes trigger the Smad pathway, whereas BMP-2-induced signaling complexes trigger an independent pathway resulting in the induction of alkaline phosphatase (ALP) activity. The latter pathway is mediated through activation of p38 MAPK.

**EXPERIMENTAL PROCEDURES**

**Materials**—Recombinant human BMP-2 was prepared as described previously (15). 9E10 (α-myc, directed against the myc tag (16)) mouse ascites was purchased from Harvard Monoclonals. HA.11 rabbit serum directed against the influenza hemagglutinin (HA) tag (17) and 12CA5 mouse ascites against this tag (α-HA) were from BAbCO. The IgG fractions were purified from mouse ascites using standard protocols (18). Anti-phospho-Smad1/5/8 antibody recognizing the phosphorylated form of Smad1, -5, and -8 was a gift from P. ten Dijke. Polyclonal antisera (rabbit) were raised against specific peptides from the BMP receptors (14). The anti-human transferrin receptor antibody (mouse monoclonal antibody B3/25) was purchased from Roche Molecular Biochemicals. Peroxidase-goat anti-mouse IgG or peroxidase-goat anti-rabbit IgG were obtained from Dianova, protein A-Sephase and protein G-Sepharose CL-4B from Sigma Chemical Co., and disuccinimidyl suberate from Pierce. Cy3-GaR and fluorescein isothiocyanate (FITC)-GaM IgG were from Jackson ImmunoResearch. The anti-phospho p38 antisemur was from Promega. The cell lines COS7 (CRL 1651) and C2C12 (CRL 1772) were purchased from American Type Culture Collection. MC3T3 and C2C12 cells were obtained from mouse ascites using standard protocols. After electrotransfer and blocking (10 mM Tris, pH 7.9, 150 mM NaCl, 5 mM KCl) at 4°C overnight, the membranes were incubated with the monoclonal antibodies 9E10 (20 μg/ml), 12CA5 (10 μg/ml) for 12 h at 4°C. Detection of adsorbed antibodies was performed by ECL (Amer sham Biosciences, Inc.), employing Peroxidase-GaM diluted 1:10,000. For detection of Smads the membrane was blocked with 10% Triton X, pH 7.9, 150 mM NaCl, 0.5% Tween 20, 3% dry milk at room temperature for 1 h, followed by incubation with α-Phospho Smad1/5/8 (1:1000 dilution) or α-Smad1/5/8 (1:1000 dilution) overnight at 4°C (20). Detection of adsorbed antibodies was performed by ECL (Amer sham Biosciences, Inc.), employing Peroxidase-GaM diluted 1:25,000 in blocking buffer. The antibody for the phosphorylated form of p38 (Promega) was used at a 1:1000 dilution. Blots were washed three times with PBS, incubated with the diluted antibody (diluted 1:2000 in TBS/0.05% Tween 20/0.1% BSA) and detection with the secondary antibody Peroxidase-GaM diluted 1:10,000 in TBST/0.1% BSA.

**Ligand Binding and Cross-linking**—BMP-2 was labeled by [125I] using the chloramine T method as described previously (21). Incubation efficiency was 96%. Before binding and cross-linking, C2C12 and MC3T3 cells were starved for 12 h in DMEM supplemented with 0.2% FCS. Confluent 10-cm plates of transfected COS cells or 70% confluent 14-cm plates of C2C12 or MC3T3 cells were incubated for 2–6 h at 4°C with 5 μg [125I]BMP-2 in KRH buffer containing 0.5% fatty acid-free BSA. For MC3T3 and C2C12 cells, 0.01% Tween 20 was added. Cross-linking was performed with disuccinimidyl suberate as described previously for TGF-β (22). Cross-linking was stopped by adding sucrose to a final concentration of 7% in KRH. Cell lysis and immunoprecipitation was performed as described above.

**Immunofluorescence Co-patching of Cell Surface Receptors**—To measure oligomerization of BMP receptor mutants directly at the cell surface, we employed antibody-mediated immunofluorescence co-patching of epitope-tagged receptors, described by us recently (14, 24, 25). COS7 cells grown on glass coverslips were co-transfected transiently using DEAE-dextran by pairs of receptors carrying different epitope tags (for example, myc-BRI-a together with HA-BRII, HA-BRII-b together with myc-TC1, or an epitope-tagged BMP receptor together with the unrelated TIR as a control) in mammalian expression vectors (pcDNA1, except for the TIR, which was in pcD). After 44–48 h, the cells were blocked with normal goat IgG (200 μg/ml, 45 min, 4°C in Hank’s balanced salt solution containing 20 μg/ml, pH 7.4, and 1% BSA) and labeled in the cold (to avoid internalization and enable exclusion of cell surface labeling) in the same buffer with primary anti-tag IgG (20 μg/ml, 45 min, rabbit HA.11 against the HA tag (BAbCO) together with either mouse α-myc (BAbCO) or mouse B3/25 against the TIR (Roche Molecular Biochemicals). This was followed by labeling/patching with secondary IgG-Cy3-GaM and FITC-GaM (20 μg/ml, 45 min, 4°C in Hank’s balanced salt solution containing 20 μg/ml, pH 7.4, and 1% BSA). The medium was replaced with PBS, 0.01% Tween 20, 4°C and mounted in SlowFade (Molecular Probes). Fluorescence digital images were recorded using a charge-coupled device camera as described (14). The FITC and Cy3 images were exported in TIFF format to Photoshop (Adobe) and superimposed. The numbers of red, green, and yellow (superimposed red and green) patches were counted on the computer screen, counting at least
RESULTS

TC3 Fails to Form Preformed Receptor Complexes with BRII—To study hetero-complex formation between BRII and BRI-a or BRI-b, we performed co-immunoprecipitation studies on COS7 cells co-transfected with a truncated HA-tagged BRII (HA-TC1, -TC3, -BRII-SF, -TC4, -TC5, -TC6, -TC7, -TC8; Fig. 1) and myc-tagged BRI-a or BRI-b. After cell lysis, BRI-a or BRI-b were immunoprecipitated with α-myc antibodies or with polyclonal antisera recognizing the receptors specifically (14). The immunoprecipitates were subjected to SDS-PAGE and Western blotting. Blots were analyzed with 8 μg of pRL-Tk (Promega) as reference for transfection efficiency and 10 μg of BMP receptor constructs (in double-receptor transfections, 5 μg of each receptor; in transfections with a single BMP receptor, 5 μg of pcDNA1-βGal replaced the second BMP receptor construct). After 7 h in complete medium, cells were starved in medium with 0.5% FCS (4–6 h) and incubated with or without 10 nM BMP-2 in low serum for 24 h. Luciferase activity was measured using a dual-luciferase assay system (Promega).

Alkaline Phosphatase Assay—C2C12 cells (1 × 10⁶ cells per well of 6-well dish) were transfected by electroporation using pBRE-luc (26) and the indicated BMP receptor constructs as described by us earlier (14). After 7 h in complete medium, cells were starved in medium with 0.5% FCS (4–6 h) and incubated with or without 10 nM BMP-2 in low serum for 24 h. Luciferase activity was measured using a dual-luciferase assay system (Promega).

Alkaline phosphatase was measured using standard protocols (27).

Fig. 2. Dissection of different BRII mutants for their ability to form preformed hetero-oligomeric complexes of BRI-a or BRII-b. A, complex formation between truncated HA-BRII and myc-BRII. COS7 cells were transiently co-transfected with HA-tagged truncations of BRII and myc-tagged BRI-a or BRI-b. After immunoprecipitation with a polyclonal antibody against BRII-b (lanes 1–4, 7, 8) or α-HA (lanes 5 and 6), Western blotting was performed using the α-HA antibody. Detection was by ECL, using G:3 IgG. All lanes were loaded with immunoprecipitates derived from the same amount of cell lysates (one 10-cm dish). HA-TC5, -TC7 appear as bands of around 150 kDa, HA-TC6 around 130 kDa, HA-TC5 near 120 kDa, HA-TC4 appears around 100 kDa, and HA-TC3 appears around 70 kDa (lanes 1–6). HA-TC3 shows no detectable preformed complex with BRII-b (lane 8). Lane 5 and 6 are controls showing the expression of HA-TC4 or HA-TC3 respectively. B, complex formation between truncated versions of HA-BRIII and myc-BRI-a. Conditions were identical to those in A except that myc-tagged BRII-b were replaced by myc-tagged BRII-a and immunoprecipitation was performed using a 9E10 (α-myc) antibody. Lane 1, the position of HA-TC1. As demonstrated in lane 1, HA-TC3 forms a preformed hetero-oligomeric complex with BRI-a.

myc-BRI-b, immunoprecipitated and blotted with the same antibody (α-HA) (Fig. 2A, lane 6). Similar experiments using BRI-a instead of BRII-b, however, show that HA-TC3 co-precipitates with myc-BRI-a (Fig. 2B, lane 1). The association of HA-TC1, which lacks nearly all of the cytoplasmic domain, could not be measured due to problems in its immunoprecipitation. However, HA-TC1 was expressed at the cell surface, as demonstrated by immunofluorescence co-patching experiments, which also enabled studies on its association with BRII receptors (see Fig. 4). The above results demonstrate the existence of preformed complexes between BRII-b or BRII-a and HA-TC4 to TC8 (Fig. 2A, lanes 1–5 and Fig. 2B, lanes 1–8). HA-TC3, however, shows a difference in hetero-oligomerization with BRII-a and BRII-b; it forms preformed complexes with BRII-a (Fig. 2B, lane 1) but fails to do so with BRII-b (Fig. 2A, lanes 6 and 8).

In a second approach, preformed complexes of TC3 with BRII-a or BRII-b were analyzed after in vivo labeling of transfected COS7 cells. The cells were co-transfected with myc-BRI-a or -BRII-b together with HA-tagged TC4, TC3, or BRII-SF (Fig. 3, lane 1–6). After labeling with [35S]methionine and [35S]cysteine, cell lysates were immunoprecipitated with α-myc antibodies. This experiment demonstrated clearly that TC3 fails to form preformed complexes with BRII-b (Fig. 3, lane

Fig. 1. Truncated BRII receptors. To determine the function of the BRII tail, eight truncated versions of BRII were generated. All receptor constructs were tagged with either HA or myc epitope tags at the N terminus. BRII-SF is a naturally occurring splice variant of BRII (5). TC3 stops immediately after the kinase domain of BRII. In TC1, the stop codon is inserted directly before the kinase domain. The position for stop codons in TC4 to TC8 is described under “Results.”

100 patches per cell on 10–15 cells in each case.

Detection of Smad and p38 Phosphorylation in MC3T3 Cells—MC3T3 cells were transfected with the indicated BMP receptor constructs. 24 h (for Smad phosphorylation) or 44 h (for phosphorylation of p38) post-transfection, cells were starved for 24 h (Smad) or 5 h (p38 phosphorylation) in DMEM supplemented with 0.5% FCS. The cells were then incubated with 20 nM BMP-2 in low serum for 30 min (Smad) or 90 min (p38) and solubilized in TNE lysis buffer (20 mM Tris, pH 7.4, 150 mM NaCl, 1% Triton X-100, 1 mM EDTA) supplemented with protease inhibitors (Boehringer complete mixture and 1 mM PMSF) and phosphatase inhibitors (50 mM NaF, 10 mM Na₄P₂O₇, and 25 mM Na₃VO₄) for 10 min on ice. Equal amounts of protein were subjected to 12.5% SDS-PAGE.

Luciferase Reporter Assay—C2C12 and MC3T3 cells (1 × 10⁶ cells per well of 6-well dish) were transfected by electroporation using pBRE-luc (26) and the indicated BMP receptor constructs as described by us earlier (14). After 7 h in complete medium, cells were starved in medium with 0.5% FCS (4–6 h) and incubated with or without 10 nM BMP-2 in low serum for 24 h. Luciferase activity was measured using a dual-luciferase assay system (Promega).

Alkaline Phosphatase Assay—C2C12 cells (1 × 10⁶ cells per well of 6-well dish) were transfected by electroporation using 8 μg of pRL-Tk (Promega) as reference for transfection efficiency and 10 μg of BMP receptor constructs (in double-receptor transfections, 5 μg of each receptor; in transfections with a single BMP receptor, 5 μg of pcDNA1-βGal replaced the second BMP receptor construct). After 7 h in complete medium, cells were starved in medium with 0.2% FCS (4–6 h). They were incubated with or without 50 nM BMP-2 in low serum for 3 days. Renilla luciferase activity was measured using the luciferase assay system from Promega. Lysates were normalized to protein content. Alkaline phosphatase was measured using standard protocols (27).
with BRI-b. In vivo titration was performed using SF, or TC4 plus tagged BRI-b or BRI-a. After cell lysis immunoprecipitated with were transfected with HA-BRI-b and HA-TC3 and immunoprecipitated with α-HA or α-myc antibodies as indicated above each lane. Lanes 1, 2, 4, and 5 show preformed receptor complexes of HA-TC4 and HA-BRII with BRI-a or BRI-b, lane 6 shows complex formation between BRI-a and TC3. As shown in lane 3 there is no detectable preformed complex of BRI-b and TC3 in the absence of ligand. BRI-b or BRI-a appear as double bands at around 55 kDa. Lanes 7, 8, and 9 are controls.

But does associate with BRI-a (Fig. 3, lane 6). Control cells were transfected with HA-BRI-b and HA-TC3 and immunoprecipitated with α-HA, showing that both proteins are highly expressed (Fig. 3, lane 9). BRI-a and BRI-b are expressed in comparable amounts in all lanes (Fig. 3, lanes 1–6). Thus, two alternative approaches based on co-immunoprecipitation have shown that TC3 fails to form preformed complexes with BRI-b but is able to do so with BRI-a.

Co-patching Studies of Cell Surface Receptors Demonstrate Defects in the Ability of TC3 and TC1 to Form Preassembled Complexes with BRI Subtypes—The experiments described above involve detergent solubilization, which might alter receptor interactions. To validate the results for the receptors at their natural cell surface environment, it was desired to measure the hetero-oligomerization of the BMP receptor mutants directly in the plasma membrane of live cells. To this end, we employed the immunofluorescence co-patching method that we have applied recently to demonstrate homo- and hetero-oligomerization of TGF-β family receptors (14, 24, 25, 28). In this method (detailed in Ref. 14) two receptors carrying different epitope tags at their extracellular termini are co-expressed at the surface of live cells. One tagged receptor is forced into micropatches by a double layer of bivalent IgGs using a fluorescent secondary antibody. The co-expressed receptor, which carries a different tag, is patched and labeled by primary antibodies from another species and secondary antibodies coupled to another fluorophore. Receptors residing in mutual oligomers will be swept into the same micropatches. If one uses red (e.g. Cy3) and green (FITC) fluorophores, mutual patches appear yellow when the two images are overlapped.

Fig. 4 (A–G) shows typical results of co-patching experiments aimed at analyzing the oligomerization of BRII, TC3, and TC1 with either BRI-a or BRI-b; because the experiments were performed in the absence of ligand, they measure preformed receptor complexes. The averaged results of such measurements on many cells are depicted in Fig. 4H. The images reveal a significant amount of co-patching (yellow patches) between BRI-a or BRI-b and BRII (Fig. 4, A, B, and H). On the other hand, control experiments to examine the level of background nonspecific co-patching of e.g. BRI-a with the unrelated human transfferrin receptor yielded a markedly lower level of co-patching (Fig. 4, G and H). A similar low level was obtained for the co-patching of the TIR with BRII (not shown). This level represents the background co-patching due to the cumulative contribution of various factors other than specific oligomeric interactions (accidental overlap of patches, co-localization due to mutual localization in other cellular subdomains, or nonspecific interactions) to the co-patching. As shown in Fig. 4H, the average percentage of myc-BRI-a found in mutual patches with co-expressed HA-BRII (and vice versa) was 32%, and an even higher percentage (41%) was found for preformed complexes of HA-BRI-b with myc-BRII. Even when the background co-patching level (averaging 18% for the control pairs) is subtracted, one is left with significant co-patching levels of 14 and 23% (for BRI-a-BRII and BRI-b-BRII, respectively).

To investigate the ability of the BRII truncation mutants TC3 (the only truncated mutant that showed a difference in preformed complexes with BRI-b) and TC1 (which is even further truncated, but could not be measured in experiments based on immunoprecipitation due to the inability of the antibodies to precipitate it) to form hetero-complexes with BRI-a and BRI-b, we studied the co-patching of each of these mutants with BRI-a and with BRI-b. The results (Fig. 4, C–H) demonstrate clear differences between the mutants and BRII, as well as between TC3 and TC1. The association of TC3 into hetero-oligomeric preformed complexes with BRI-a is just as efficient as that of BRII (no significant difference between their co-patching levels with BRI-a; p > 0.05 according to Student’s t test) but is nearly completely lost (only a few percent above the background co-localization level) for the pair TC3-BRII (Fig. 4, D and H). Note that the co-patching level of TC3 with BRI-a is significantly higher than that of TC3 with BRI-b (p < 0.001). As for TC1, this most extreme truncation mutant showed only a marginal level of co-patching even with BRI-a, and no co-patching at all above the experimental error with BRI-b.

All BRII Truncations Bind BMP-2 following Complex Formation with BRI Subtypes—As demonstrated above, TC3 fails to form preformed complexes with BRI-b but still forms complexes with BRII. TC1, the shortest BRII mutant, is not present in preformed complexes neither with BRI-a nor with BRI-b. To investigate the oligomerization pattern of the truncation mutants and BRI-a or BRI-b in the presence of ligand, we performed ligand binding and cross-linking experiments (Fig. 5). COS7 cells were transiently transfected with either the HA-tagged BRII truncation mutants alone or in the presence of HA-tagged BRI. Binding and cross-linking experiments were performed as described earlier (14). As shown in Fig. 5, ligand-bound hetero-oligomers between all the BRII truncation mutants and BRI-a (Fig. 5A) or BRI-b (Fig. 5B) were detected. If the mutants were transfectected alone, no binding of 5 nM 125I-BMP-2 was detected (data not shown). The existence of a hetero-oligomeric ligand-dependent complex between TC3 and BRI-b was confirmed by co-transfection of untagged BRI-b and TC3 (Fig. 5B, lane 10). Importantly, TC1, which did not associate with either BRI-a or BRI-b in the absence of ligand, was distinctly cross-linked to ligand when co-expressed with either of the BRII subtypes. The fact, that co-expression with BRI-b or BRI-a enabled TC3 and TC1 to be cross-linked to BMP-2, suggests the formation of hetero-complexes between these receptors. Because hetero-complexes of BRI-b/TC3 and between both BRII subtypes and TC1 were detected only in the presence of ligand, we distinguish between “BMP-2 induced complexes” and “preformed complexes.”

Signaling via the Smad Pathway Correlates with Preformed Heteromeric BMP Receptor Complexes—Because signaling was measured in C2C12 and MC3T3 cells, we confirmed that they
co-transfected receptors are designated by the representative panels (analyzed by counting the numbers of overlapped.

Superimposed red and green images were used to count the numbers of red- and green-labeled receptors appear yellow when the two fluorescent charge-coupled device images are overlapped. Bar, 20 \mu m. A, HA-BRII (red) and myc-BRI-a (green) show a significant level of co-patching above the control (14% over the background level; p < 0.001 according to Student's t test). B, HA-BRI-b (red) and myc-BRII (green) exhibit a significant co-patching level (23% above the control; p < 0.001). C, HA-BRI-a (red) and myc-TC3 (green) exhibit a co-patching level similar to that of wt BRII with BRI-a, which is significantly higher than the control (17% above the background level; p < 0.001). D, HA-BRI-b (red) and myc-TC3 (green) co-patching is only slightly above the background level (7%; p < 0.001). E, HA-BRI-a (red) and myc-TC1 (green) co-patching is only slightly above the background co-patching level (7%; p < 0.001). F, HA-BRI-b (red) and myc-TC1 (green) do not co-patch significantly above the control (p > 0.1). G, HA-BRI-a (red) and TIR-green show a low degree of co-patching. This level (18%; see panel H) is the background percentage of co-patching when unrelated receptors are employed. Similar levels were obtained for co-patching of the TIR with other receptors (BRII or the type II TGF-\beta receptor). H, quantification of the co-patching data. The pairs of co-transfected receptors are designated by the letters corresponding to the representative panels (A-G).

FIG. 4. Immunofluorescence co-patching to explore heteromeric complexes of BRII, TC3, and TC1 with BRII-a or BRII-b at the cell surface. COS7 cells were co-transfected transiently with pairs of receptors carrying different epitope tags as indicated below for each panel. Live cells were labeled in the cold consecutively by a series of antibodies to mediate patching and fluorescent labeling, as detailed under "Experimental Procedures." This labeling protocol results in HA-tagged receptors labeled by Cy3 (red), whereas myc-tagged receptors or the TIR (control) labeled by FITC (green); mutual patches containing both red- and green-labeled receptors are designated by the representative panels (analyzed by counting the numbers of overlapped. Bar, 20 \mu m. A, HA-BRII (red) and myc-BRI-a (green) show a significant level of co-patching above the control (14% over the background level; p < 0.001 according to Student's t test). B, HA-BRI-b (red) and myc-BRII (green) exhibit a significant co-patching level (23% above the control; p < 0.001). C, HA-BRI-a (red) and myc-TC3 (green) exhibit a co-patching level similar to that of wt BRII with BRI-a, which is significantly higher than the control (17% above the background level; p < 0.001). D, HA-BRI-b (red) and myc-TC3 (green) co-patching is only slightly above the background level (7%; p < 0.001). E, HA-BRI-a (red) and myc-TC1 (green) co-patching is only slightly above the background co-patching level (7%; p < 0.001). F, HA-BRI-b (red) and myc-TC1 (green) do not co-patch significantly above the control (p > 0.1). G, HA-BRI-a (red) and TIR-green show a low degree of co-patching. This level (18%; see panel H) is the background percentage of co-patching when unrelated receptors are employed. Similar levels were obtained for co-patching of the TIR with other receptors (BRII or the type II TGF-\beta receptor). H, quantification of the co-patching data. The pairs of co-transfected receptors are designated by the letters corresponding to the representative panels (A-G).

Superimposed red and green images were used to count the numbers of red, green, and yellow patches (counting at least 100 patches per cell on 10–15 cells in each case). The % co-patching (percentage of a given tagged receptor in mutual patches with the other receptor) is given by: 100 × [yellow + green] / [red + green], where red-labeled receptors and 100 × [yellow + green] / [green-labeled receptors (14). Because these values were very close, a single value is depicted for each pair. The values shown are mean ± S.E. of the % co-patching of the different receptor pairs.

FIG. 5. Detection of cell surface BMP-receptor hetero-complex formation by ligand binding and cross-linking. COS7 cells were co-transfected with pairs of epitope-tagged receptors. After binding and cross-linking of \textsuperscript{125}I-BMP-2 (5 nM), immunoprecipitation was performed with 12CA5 (\alpha-HA). Immunoprecipitates derived from the same amount of cell lysates (one 10-cm dish) were loaded on each lane of the gel and analyzed by SDS-PAGE and autoradiography. A, lanes 1–8 depict complexes of HA-BRI-a (black arrow) and HA-tagged truncation mutants of BRII (triangle). Binding to all receptors can be detected. B, lanes 1–8 depict complexes of HA-BRI-b (black arrow) and HA-tagged BRII truncation mutants or BRII (triangle). HA-BRI-b complexes with HA-BRII are shown in lane 9. To confirm binding of TC3 in the presence of BRII-b to BMP-2, co-transfection of TC3 with untagged BRII-b and immunoprecipitation of TC3 were performed. Lane 10 as well as lane 2 depict complexes of TC3 and BRII-b. The BRIII truncation mutants and BRII alone show no binding to 5 nM \textsuperscript{125}I-BMP-2 (not shown).

We analyzed BMP-2 signaling in C2C12 and MC3T3 cells by the use of the Smad binding element-driven luciferase reporter pSBE-luc (26). Both cell lines respond to BMP-2 by differentiating into osteoblasts (27, 29, 30). Activation of the Smad pathway is induced by BMP-2 as seen in Fig. 7 (\beta-Gal as mock-control in A and B). We investigated the effect of various BMP-receptor constructs on activation of pSBE-luc.

Single transfection with either BRII or BRII-SF resulted in increased luciferase activity upon BMP-2 stimulation (Fig. 7A in C2C12 cells; Fig. 7B in MC3T3 cells). Transfection with BRII-a or BRII-b, however, showed no increase in activity in response to BMP-2, unless BRII or BRII-SF were co-transfected (data not shown). This suggests that BRII is limiting in these cells.

Next we analyzed the effect of BRIII mutants on the activation of the Smad reporter. Interestingly, ectopic expression of
HA-TC1, a truncated BRII lacking the complete kinase domain, had no effect on the pSBE-luc response (Smad pathway signaling), which remained similar to that observed in mock-transfected cells (Fig. 7A). Because TC1 lacks kinase activity, it would inhibit Smad signaling if it were able to compete with endogenous BRII for BRI receptors. The lack of the effect of TC1 correlates with its inability to form preformed complexes with BRI-a and BRI-b (Figs. 2 and 3) and contrasts with its ability to form BMP-induced complexes with them (Fig. 4), suggesting that the Smad response to BMP-2 is mediated via the preformed complexes and not via the BMP-induced complexes. Accordingly, BRII-KR (a kinase-dead mutant that differs from wt BRII in a single point mutation, K230R) overexpression inhibited BMP-2 signaling via the Smad pathway in a dominant-negative fashion (Fig. 7B). Because the BRII-KR point mutant is similar to the wt BRII in the ability to form preformed complexes (as well as BMP-2-induced complexes) with BRI-a or BRI-b (data not shown), these findings demonstrate that dominant-negative effects on the Smad pathway correlate with the ability of the kinase-negative mutants (TC1 or BRII-KR) to form preformed complexes with BRI-a and BRI-b.

Transfection of C2C12 cells with HA-TC3 did not alter the transcriptional activation of pSBE-luc in response to BMP-2, whereas a slight increase in the response was observed in MC3T3 cells (Fig. 7, A and B). Because the Smad response appears to be mediated via preformed receptor complexes (see above results with TC1), this suggests that TC3, which forms preformed complexes only with BRI-a (Figs. 2 and 3), is only partially active even in the complex with BRI-a.

BRII-KR but Not TC1 Has a Dominant-negative Effect on the Smad Pathway—To validate that the results obtained in the experiments on pSBE-luc transcriptional activation indeed reflect activation of the Smad pathway by BMP-2, we examined directly the effects of transfection of BRII and the key mutants (TC1 and BRII-KR) on the phosphorylation of the BMP R-Smads (Smad1, -5, and -8) in response to BMP-2. MC3T3 cells transfected with the indicated receptors were lysed after stimulation with 20 nM BMP-2 for 30 min, and cell lysates were subjected to SDS-PAGE and Western blotting (Fig. 7C). Smad phosphorylation was analyzed using an antibody that specifically detects phosphorylated BMP R-Smads (see “Experimental Procedures”). Transfection with HA-BRII strongly elevated the phosphorylation of BMP R-Smads upon BMP-2 treatment (Fig. 7C, lane 2). On the other hand, ectopic expression of TC1 had no effect on Smad phosphorylation in response to BMP-2 (Fig. 7C, lane 4), in accord with the inability of TC1 to form preformed receptor complexes with BRI-a or BRI-b (Figs. 2 and 3) and with its lack of effect on the transcriptional activation of the pSBE-luc reporter (Fig. 7, A and B). On the other hand, BRII-KR expression reduced Smad phosphorylation to about...
A

![Graph A]

FIG. 8. TC1 acts dominant-negative in the p38 MAPK/ALP pathway. A, C2C12 cells were transfected with various BRII truncation mutants or mock as control. ALP activity was measured after 3 days cultivation of cells in 0.2% FCS and 10 nM BMP-2. Error bars represent S.D. from three different measurements. Transfection with BRII-SF and BRII increased ALP induction by BMP-2. Transfection of TC3 showed no effect. TC1 had a dominant-negative effect on ALP induction by BMP-2. B, MC3T3 cells were transfected with the indicated BMP receptor constructs. Serum-starved cells were stimulated with 20 nM BMP-2 in low serum for 90 min. After protein determination, equal amounts of protein were subjected to 12.5% SDS-PAGE and transferred to nitrocellulose membrane. Phosphorylated p38 MAPK was detected using α-Phospho-p38 antiserum. Transfection with BRII-KR resulted in a significant decrease in p38 phosphorylation in response to BMP-2. TC1 transfection mediated a nearly complete block of p38 MAPK phosphorylation in response to BMP-2, suggesting that TC1 has a dominant-negative effect on the p38 MAPK pathway.

50% (Fig. 7C, lane 6), in accord with its dominant-negative effect on the Smad pathway response (Fig. 7B). These findings validate in a direct assay that BRII-KR but not TC1 inhibits the Smad pathway in a dominant-negative manner.

Activation of Alkaline Phosphatase Is Mediated upon Stimulation of BMP-2-induced Receptor Complexes—Aside from activating the Smad pathway, BMP receptors can activate ALP production by a presumably different pathway. To investigate whether this pathway is activated in a similar manner to the Smad pathway, we measured ALP activity in C2C12 cells transfected with various BRII mutants (Fig. 8A). ALP activity was induced upon stimulation with BMP-2; this response was elevated in C2C12 cells transfected with either BRII or BRII-SF (Fig. 8A). Transfection with TC3 neither increased nor reduced signaling compared with endogenous receptor signaling, in accord with the notion that it is a partially active mutant (see Fig. 7B). On the other hand, TC1 (which lacks almost the entire cytoplasmic tail, including the kinase domain) had a dominant-negative effect on the induction of ALP activity by BMP-2. The dominant-negative phenotype of TC1 in the ALP pathway is fully correlated with its ability to form BMP-2-induced complexes with BRII-a and BRII-b (Fig. 5) and suggests that the induction of ALP in response to BMP-2 is mediated via these complexes. This contrasts with the lack of effect of TC1 on Smad signaling, presumably due to its inability to form preformed complexes with both BRII subtypes.

Activation of p38 MAPK by BMP-2 Is Triggered through BMP-2-induced Receptor Complexes—To examine whether p38 MAPK might be involved in the pathway leading to ALP induction, we transfected MC3T3 cells with different BRII-receptor constructs and examined p38 activation using Western blotting with anti-phospho-p38 antiserum. Transfection with BRII variants that activate the Smad pathway (BRII-SF, BRII) did not influence the phosphorylation of p38 in response to BMP-2 (Fig. 8B, lanes 7–10). Transfection with BRII-KR led to a decrease in p38 phosphorylation in response to BMP-2 (Fig. 8B, lanes 11 and 12) indicating the relevance of the kinase domain of BRII for p38 phosphorylation. Interestingly, transfection with a BRII mutant lacking most of the cytoplasmic region (TC1) resulted in a nearly complete elimination of p38 phosphorylation in response to BMP-2 (Fig. 8B, lanes 5 and 6). As shown in Fig. 5 (A and B), TC1 can be recruited into BMP-2-induced complexes and thus can block signaling pathways initiated by these complexes. This is in accord with the demonstration (Fig. 8, A and B) that TC1 has a dominant-negative effect on both p38 phosphorylation and ALP activation by BMP-2 and indicates that p38 MAPK is an upstream component of the ALP pathway.

DISCUSSION

BMP receptors exhibit a very flexible oligomerization pattern at the cell surface, including a significant level of preformed heteromeric BRII-BRII complexes (14). The ligand BMP-2 has at least two different options to initiate signal transduction via these receptors: 1) binding to preformed receptor complexes and inducing a conformational change that activates this complex; 2) binding to the high affinity BRII receptors, subsequently recruiting unliganded BRII into the ligand-mediated signaling complex.

In this study, we examined the possibility that preformed and BMP-2-induced complexes, both hetero-complexes of the BMP receptors, activate different signaling pathways. The use of BRII truncation mutants, we were able to map receptor domains within the cytoplasmic tail region of the receptor, which are important for the ability of BRII to form heteromeric complexes with BRI in the absence of ligand. TC3, which is 29 amino acids shorter than the naturally occurring BRII splice variant BRII-SF (Fig. 1), formed preformed complexes with BRI-a but failed to do so with BRI-b (Figs. 2–4). TC1, which lacks the entire kinase domain, did not form preformed complexes with either BRI-a or BRI-b. This shows that the region above 29 amino acids is essential for the association of BRII-SF with BRI in preassembled complexes and indicates that the interactions of BRI-a and BRI-b with BRII in the absence of ligand are not identical. On the other hand, all the BRII mutants (including TC3 and TC1) were effectively recruited by BMP-2 into heteromeric receptor complexes with both BRII subtypes (Fig. 5). The difference between the abilities of some of the mutants (TC1 and TC3) to form preassembled complexes as compared with their capability to be recruited into BMP-2-induced complexes enabled us to analyze whether these complexes mediate different signaling pathways. The experiments with TC1 were especially informative, because the ability of this kinase-negative mutant to induce a dominant-negative effect on signaling originating from BMP-2-induced complexes strictly correlated with its ability to form heteromeric complexes with BRII receptors. To acquire a dominant-negative character, an inactive receptor mutant (in this case, TC1) should be able to compete with the endogenous receptors (BRII) for binding to their signaling partners (in this case, BRII subtypes). The inability of TC1 to form preformed complexes with either BRI-a or BRI-b correlates with its lack of effect on Smad signaling (Fig. 7), whereas its dominant inhibitory effect on ALP induction as well as on p38 MAPK activation by BMP-2 correlates with its ability to be recruited into BMP-2-induced complexes with BRI-a and BRI-b (Fig. 8). This suggests that the two responses (activation of the Smad pathway and induc-
Following the binding of ligand (via p38 MAPK) is not sufficient to mediate different signaling. Activation of different signaling pathways requires that the final signaling complexes will be dissimilar. Thus, our findings suggest that the ligand-bound preformed and BMP-2-induced complexes of the BMP receptors are distinct from each other. There are several possible mechanisms that can give rise to such differences. For example, the majority of the BMP receptors (both BRII and BRI) do not reside in homomeric oligomers in the absence of ligand (12), and the preformed complex is therefore likely to be a heterodimer containing one subunit of each receptor type. On the other hand, BMP-2 binding increases the homo-oligomerization of BRI but not of BRII (because the latter does not bind ligand effectively in the absence of BRI) (12). Thus, it is possible that BMP-2-induced complexes contain an oligomeric BRI complexed with one BRII subunit and are therefore physically different from ligand-bound preformed complexes. Moreover, the different complexes (preformed and BMP-2-induced) may interact with additional proteins, which would be recruited selectively into one type of complex but not into the other. Potential candidates are XIAP (36), BRAM-1 (37), and FKBP12 (38) (for review see Ref. 13). Interestingly, the activation of the Smad 2/3 pathway by other members of the TGF-β receptor family (TGF-β and activin receptors) appears to follow ligand-mediated receptor hetero-oligomerization (39) rather than signaling through preformed receptor complexes; at least in the case of the TGF-β receptors, the amount of preformed heteromeric complexes is very low (25). The dominant-negative effect of a truncated BRII lacking the cytoplasmic part has been demonstrated before in Xenopus, where the expression of this mutant leads to a conversion of ventral to dorsal mesoderm during embryogenesis (40, 41). It has been shown that BMP-2 stimulates the MAPK pathway by a BMP-2-induced up-regulation of mRNA for ERK1 and ERK2 during osteoblastic differentiation of C3H10T1/2 cells; both MAPKs are involved in the induction of ALP activity (42). Interestingly, ERK activation was shown to inhibit nuclear translocation of Smad1, thus blocking the Smad pathway (43). This may facilitate cross-talk between the two BMP-2 pathways (initiated by preformed and BMP-2-induced complexes), where one long term effector (ERK) of the Smad-independent pathway (via BMP-2-induced receptor complexes) antagonizes the fast BMP response (mediated via preformed complexes). p38 MAPK was suggested to be a component of the BMP signaling pathway (12, 44). As we show here, p38 MAPK is activated through the Smad-independent pathway. Other potential signaling molecules for this pathway are the collagen integrin receptors (34).

In conclusion, we show that the initiation of different BMP-2 signaling pathways is strictly dependent on the mode of oligomerization of the same types of receptors, BRII and BRI. Although activation of the Smad pathway is initiated by the binding of BMP-2 to preformed BRI-BRII complexes, a different pathway culminating in the induction of ALP through p38 MAPK is activated by the recruitment of these receptors into ligand-mediated heteromeric complexes (Fig. 9). Further studies are needed to elucidate the composition of the signaling receptor complexes and the initial steps of activation of the latter pathway.

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