Bone morphogenetic protein-2 (BMP-2) is an important regulator of osteoblast differentiation. However, the regulation of osteoblast apoptosis by BMP signaling remains poorly understood. Here we examined the role of type I BMP receptor (BMP-RI) in osteoblast apoptosis promoted by BMP-2. Despite undetectable BMP-RI expression in OHS4 cells, BMP-2 or BMP-2 overexpression increased osteoblast differentiation similarly as in SaOS2 cells which express BMP-RI, as shown by alkaline phosphatase and CBFA1/RUNX2 expression. In contrast to SaOS2 cells, however, BMP-2 or BMP-2 overexpression did not increase caspase-9 and caspases-3, -6, and -7 activity and DNA fragmentation in OHS4 cells. Consistently, BMP-2 increased protein kinase C (PKC) activity, and PKC inhibition suppressed BMP-2-induced caspase activity in SаOS2 but not in OHS4 cells that lack BMP-RI. A dominant negative BMP-RI inhibited BMP-2-induced caspase activity, whereas wild-type BMP-RI promoted caspase activity induced by BMP-2 in SaOS2 and MC3T3-E1 cells. Wild-type BMP-RI rescued the apoptotic response to BMP-2, and a constitutively active BMP-RI restored the apoptotic signal in OHS4 cells, supporting an essential role for BMP-RI in osteoblast apoptosis. We also assessed whether BMP-2-induced apoptosis occurred independently of osteoblast differentiation. General inhibition of caspasess did not abolish BMP-2-induced alkaline phosphatase and CBFA1/RUNX2 expression in SaOS2 cells. Furthermore, broad caspases inhibition increased matrix mineralization but did not reverse the BMP-2 effect on mineralization in MC3T3-E1 cells. These results indicate that BMP-2-induced apoptosis was mediated by BMP-RI in osteoblasts and occurred independently of BMP-2-induced osteoblast differentiation, which provides additional insights into the dual mechanism of BMP-2 action on osteoblast fate.

Bone morphogenetic proteins (BMPs) are members of the transforming growth factor-β superfamily that play essential roles in skeletal development and repair (1, 2). BMPs are involved in embryonic and post-natal osteogenesis by stimulating the commitment of mesenchymal cells into the osteoblastic lineage and promoting osteoblast differentiation (3–5) in part by increasing the expression of CBFA1/RUNX2, a transcription factor that is essential for osteoblast genes expression (6). BMP-2 promotes expression of the osteoblast phenotypic genes alkaline phosphatase (ALP), type I collagen and osteocalcin in murine preosteoblastic cells (7–9), and human osteoblastic cells (10–13). BMPs signal through type I (BMP-RIA and BMP-RIB) and type II (BMP-RII) serine/threonine kinase receptors (14–17). Binding of BMPs to BMP receptors results in BMP-RI receptor phosphorylation by BMP-RI receptor and phosphorylation of downstream Smad proteins (18, 19). BMPs activate Smad1, Smad5, and Smad8, which upon phosphorylation can associate with Smad4 in a heterodimeric complex that is translocated to the nucleus where it activates transcription (20–22). In addition to the Smad pathway, ligand binding to BMP receptors can activate other signaling pathways in osteoblasts, including mitogen-activated protein kinases (23, 24), phosphatidylinositol 3-kinase/Akt (25), and PKC (26).

BMP-RIA and BMP-RIB are expressed in a variety of osteoblastic cells (3) and play major roles in osteogenesis (27, 28). Studies using truncated kinase-inactivated forms and constitutively active forms of BMPRs demonstrated that BMP-RIs mediate BMP signaling during osteoblast differentiation (3, 9, 29–31). In preosteoblastic or preadipocytic cell lines, BMP-RIA and BMP-RIB signaling stimulates osteoblast differentiation (31–33). BMP-RIB was found to be essential for the differentiation of multipotent C2C12 cells (34) and mouse calvaria cells (30) into osteoblasts. Recent data indicate that overexpression of a truncated BMP-RIB targeted to osteoblasts using the type I collagen promoter results in defective bone formation in homozygous transgenic mice, also indicating that BMP-RIB plays an important role in osteoblast commitment and bone formation (35).

The terminal maturation of osteoblasts is followed by the occurrence of apoptosis, which begins to occur during the late differentiation stage (36, 37). The importance of apoptosis for osteoblast function and bone formation has been demonstrated during bone development and remodeling (37, 38). In the developing limb, BMPs induce apoptosis of mesenchymal cells, which is critical for normal skeletal development (39–43). This effect is not limited to mesenchymal cells during development because we recently showed that BMP-2 activates apoptosis in postnatal human osteoblastic cells (26). However, the regulation of osteoblast apoptosis by BMP signaling remains poorly understood. Type IB BMP receptor was found to be involved in...
the regulation of embryonic cell death during development (40). Although this suggests that type I BMP-Rs may be involved in apoptosis, this has not been examined previously in osteoblasts. Furthermore, although BMP-2 may exert a dual control of osteoblast fate through regulation of differentiation and apoptosis, it is unknown whether BMP-2-induced apoptosis can occur independently of its effects on osteoblast differentiation.

In this study, we examined the role of type I BMP receptors in the control of apoptosis by BMP-2 in osteoblastic cells by using human osteoblastic cells lacking BMP-RIB as a model. We also tested the hypothesis that the effect of BMP-2 on osteoblast apoptosis may occur independently of its effect on osteoblast gene expression. Our results suggest that BMP-RIB is essential for BMP-2-induced activation of apoptosis in osteoblastic cells and mediates apoptosis independently of BMP-2-induced osteoblast differentiation.

**EXPERIMENTAL PROCEDURES**

**Cell Culture and Treatments**—We studied two human osteosarcoma cell lines (OHS4 and SaOS2) that express osteoblast phenotypic characteristics including alkaline phosphatase, type I collagen, and osteocalcin (44–46). We took advantage of these cell lines because they express BMP-RIB differently (see under “Results”). We also used clonal normal mouse calvaria-derived MC3T3-E1 cells that display all characteristics of differentiating osteoblasts, including mineralization in vitro (47). For analysis of mineralization, ALP activity was measured. The data are the mean ± S.E. of six values. *, p < 0.05 versus untreated controls. **Results**—After 48 h of treatment with BMP-2 (50 ng/ml), total cellular RNA was subjected to RT-PCR analysis, and amplified products were hybridized with 32P-labeled internal probes. C, aliquots of OHS4 and SaOS2 cell lysates were prepared, and BMP-RIB protein levels were determined by Western blotting and visualized by enhanced chemiluminescence method. β-Actin was used as control of protein loading.

![Fig. 1](image1.png) **Fig. 1.** Distinct expression of BMP receptors in human OHS4 and SaOS2 osteoblastic cells. Total cellular RNA was subjected to RT-PCR analysis and amplified products for Smad1, Smad4, Smad5, and Smad8, or GAPDH (A) or for BMP-RIA/ALK-3, BMP-RIB/ALK-6, BMP-RIL, or GAPDH (B) were hybridized with γ-32P-labeled internal probes. C, aliquots of OHS4 and SaOS2 cell lysates were prepared, and BMP-RIB protein levels were determined by Western blotting and visualized by enhanced chemiluminescence method. β-Actin was used as control of protein loading.

![Fig. 2](image2.png) **Fig. 2.** BMP-2 promotes osteoblast gene expression independently of BMP-RIB expression. OHS4 and SaOS2 cells were treated with increasing doses of BMP-2 or the solvent. A, after 48 h of treatment, ALP activity was measured. The data are the mean ± S.E. of six values. *, p < 0.05 versus untreated controls. B, after 24 h of treatment with BMP-2 (50 ng/ml), total cellular RNA was subjected to RT-PCR analysis, and amplified products were hybridized with γ-32P-labeled internal probes. Densitometric analysis of transcripts for ALP, Cbfa1/Runx2, and N-cadherin visualized by autoradiography was determined, and the level of expression was normalized to GAPDH. C, after 48 h of treatment with BMP-2 (50 ng/ml), equal aliquots of cell lysates were prepared, and Cbfa1 and N-cadherin protein levels were determined by Western blotting and visualized by enhanced chemiluminescence method. β-Actin was used as control of protein loading. Results shown are representative of three independent experiments.

**Analysis of Mineralization**—For analysis of mineralization, MC3T3-E1 cells that express BMP-RIA and RIB receptors (49) were cultured at confluence in Dulbecco’s modified Eagle’s medium with 10% FCS and then cultured in the presence of ascorbic acid (25 mg/ml) and inorganic phosphate (3 mM) to induce collagenous matrix release and mineralization (47). The cells were treated with Z-VAD-fmk (20 μM) or its solvent (Me2SO), in the presence or absence of BMP-2 (50 ng/ml). After 14 days, matrix mineralization was detected by alizarin red and von Kossa stainings which reveal calcium and phosphate, respectively (50), and microphotographed using an Olympus microscope (Japan). Quantification of matrix mineralization was determined by colorimetric measurement of calcium, as described (50).

**Plasmids and Transfection**—Rat cDNA for BMP-2 was kindly provided by Drs. Di Chen and G. Mundy (51). Wild-type (wt)BMP-RIB, constitutively active (ca)BMP-RIB, and dominant negative (DN)-BMP-RIB vectors were kindly provided by Dr. S. Roman-Roman (Aventis, Romainville, France). The DN-BMP-RIB construct was created by a single amino acid change in the ATP-binding site (Lys-231 to Arg) as described (40). The cBMP-RIB was created by a point mutation (Gln-203 to Asp) (40). We performed transient transfections allowing expression of the transfected cDNA for at least 48 h. Stable transfections with these vectors could not be conducted because the cells die by apoptosis. Transient transfection was sufficient to determine the changes in apoptosis induced by BMP-2 occurring at 24–48 h of treatment (26). OHS4 and SaOS2 cells were transfected with wtBMP-RIB,
BMP-RIB Mediates BMP-2-induced Osteoblast Apoptosis

Fig. 3. BMP-2 induces apoptosis in SaOS2 but not in OHS4 cells lacking BMP-RIB. A, serum-deprived OHS4 and SaOS2 cells were treated with BMP-2, the solvent, or the DNA-damaging agent etoposide (50 μM) for 24 h and then stained with TUNEL, and the percentage of TUNEL-positive cells was counted. B, OHS4 and SaOS2 cells were treated with BMP-2 (50 ng/ml), and caspase-2 and -8 activities were determined. C, OHS4 and SaOS2 cells were treated with BMP-2 (50 ng/ml) and caspase-9 and -3, -6, and -7 activities were determined. The data are the mean ± S.E. of six values. *, p < 0.05 versus untreated cells.

cBMP-RIB, and DN-BMP-RIB vectors. MC3T3-E1 cells were also transfected with wtBMP-RIB or DN-BMP-RIB vectors to ensure reproducibility of the results in normal osteoblastic cells. All cells were plated at 5,000 cells/cm² the day before transfection. The cells were co-transfected with the plasmid (15 μg/100-mm dish) and pSV-β-galactosidase control vector (Promega), at a 10:1 ratio, by calcium-phosphate precipitation according to standard procedures described by the manufacturer (Promega). After 16 h, the transfection medium was replaced with fresh medium (1% BSA, serum-free) overnight. The efficiency of transfection was controlled by determination of β-galactosidase activity. The number of β-galactosidase-positive cells was counted 72 h post-transfection. Efficient transfection of BMP-2 cDNA was also tested by the expression of BMP-2 by Western blot analysis. Apoptosis and the activity of caspases were determined as described below.

RT-PCR Analysis—The expression of BMP-RIA, BMP-RIB, BMP-RII, Smad1, -4, -5, and -8, ALP, CBFA1/RUNX2, and N-cadherin mRNA levels were analyzed by reverse transcription-PCR. Optimization of RT-PCR results was carried out by generating saturation curves of RT-PCR products of each gene and of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) against cycle number (0–35 cycles) as described (13). We chose the same cycle number (30 cycles) for all genes, except for N-cadherin and GAPDH (25 cycles), in which the amplification was linear. After treatment with BMP-2 (50 ng/ml) or the solvent, the cells were washed with PBS and lysed with Extract-All (Eurobio, France) reagent according to the manufacturer’s instructions. Three μg of total RNA from each sample was reverse-transcribed, and the cDNA samples were then divided and amplified using specific primers (20 pmol/μl tube) (13). Primers were as follows: sense 5’-TGTACCTTCCTCTGTTGCTG-3’, antisense 5’-TGGTTCACTCATAAGCAACGC-3’ for Smad1; sense 5’-CCTTACTGGAGATGTAACTG-3’, antisense 5’-CGGCATCCCCCATCTCTGATAAGG-3’ for Smad4; sense 5’-CTTGCTGACAGACCATATCC-3’, antisense 5’-CATCCCGATACCACTCCCTCC-3’ for Smad5; sense 5’-AGATGCTGGTGTGACCTAC-3’, antisense 5’-GTTGAAACTCTGCAGGCCTGC-3’ for Smad8; sense 5’-TAGAGTTTCTTCCCTCCGATGG-3’, antisense 5’-GCATACATCTGGGGATGTTTCTG-3’ for BMP-RIA; sense 5’-GCCAGACAGAGGTATTTGTTG-3’, antisense 5’-TTCCTAGACACTCATTACACT-3’ for BMP-RIB; and sense 5’-ACGGGAGAAGACAGGACACCT-3’, antisense 5’-CTAGATCAAGAGGGTCTTG-3’ for BMP-RII. Other primers were as described previously (13, 26). Southern blots were performed by running aliquots of amplified cDNA on 1.2% agarose gel followed by transfer onto nylon membrane according to the manufacturer’s protocol. Hybridization of blots was carried out overnight at 50 °C with [γ-32P]ATP-labeled internal primers. Membranes were washed twice in 2× SSC, 0.1% SDS at room temperature for 10 min, once in 0.1× SSC, 0.1% SDS at 50 °C for 10 min and then the filters were exposed to x-ray films. Autoradiographic signals were quantified using a scanner densitometer (Transydine General Corp., Ann Arbor, MI). The signal for each gene was corrected for GAPDH.

Western Blot Analysis—Cells (10,000/cm²) treated with BMP-2 (50 ng/ml) or the solvent were washed twice with cold PBS and scraped in 1 ml of ice-cold lysis buffer (10 mM Tris-HCl, 5 mM EDTA, 150 mM NaCl, 30 mM sodium pyrophosphate, 50 mM NaF, and 1 mM Na3VO4) containing 10% glycerol and protease inhibitors (Roche Applied Science). Protein samples were solubilized in 4× Laemmli SDS loading buffer and boiled at 95 °C for 5 min. Fifty micrograms of proteins, determined using the DC Protein assay (Bio-Rad), were resolved on 12% acrylamide gel and then transferred onto polyvinylidene difluoride-Hybond-P membranes (Amersham Biosciences). Blots were saturated overnight with 1% blocking solution (Roche Applied Science) in TBS buffer (50 mM Tris-HCl, 150 mM NaCl) and 0.1% Tween 20. Membranes were then incubated with mouse monoclonal anti-Cbfa1/Osf2 (92), monoclonal anti-N-cadherin (Sigma) (13), polyclonal anti-β-actin (Sigma), anti-BMP-2/4 or monoclonal anti-BMP-RIB (Santa Cruz Biotechnology) in 0.5% blocking buffer. After 1 h at room temperature, the membranes were washed twice with TBS, 0.1% Tween 20, and 0.5% blocking buffer and incubated for 1 h with horseradish peroxidase-conjugated secondary antibody for 1 h at room temperature. Following incubation with the appropriate secondary antibodies, the membranes were washed, and the signals were visualized with ECL+ blotting substrate (Amer sham Biosciences). The specific bands on the autoradiograms were quantitated by densitometry, and the level of expression was normalized to β-actin.

Detection of Apoptosis—To detect apoptosis, DNA cleavage was assessed by the TUNEL assay as described by the manufacturer (Roche Applied Science). SaOS2 and OHS4 cells (5,000/cm²) cultured on Labtek chambers in serum-deprived conditions (1% BSA, serum-free) for 24 h were treated with BMP-2 (50 ng/ml) or the solvent for 24 h and then fixed with paraformaldehyde at room temperature for 5 min. Endogenous peroxidase was quenched with 3% H2O2, and the cells were permethylated with 0.1% Triton X-100, at 4 °C for 2 min and incubated for 1 h at 37 °C with the TUNEL reaction mixture containing the terminal deoxynucleotidyltransferase. Incorporated fluorescein was detected by sheep anti-fluorescein antibody conjugated with horseradish peroxi-
dase. The TUNEL signal was detected with peroxidase-labeled antidigoxigenin antibody revealed with diaminobenzidine and mounted. TUNEL-positive cells were detected by brown nuclei and nuclear fragmentation. Positive controls consisted of cells treated for 24 h with 50 μM etoposide, a topoisomerase II inhibitor that induces DNA damage and nuclear fragmentation associated with apoptosis. Additional positive controls consisted of cells treated with ENase I for 10 min. Negative controls were obtained by omitting the transferase from the reaction (26). In each experiment, the number of total and TUNEL-positive cells was counted. A total of 1,500 cells was counted for each cell type, and the results were expressed as percentage of total cells.

**Determination of Caspase Activity**—To identify the caspases involved in BMP-2-induced apoptosis, cells (10,000/cm²) were cultured in 1% BSA/serum-free medium in the presence of BMP-2 (50 ng/ml) or the solvent for 24 h and then treated with BMP-2 (50 ng/ml) or the solvent for 10–60 min. The cells were lysed in lysis buffer (25 mM Tris-HCl, pH 7.4, 0.5 mM EDTA, 0.5 mM EGTA, 0.05% Triton X-100, 10 mM β-mercaptoethanol, 1 μg/ml leupeptin, 1 μg/ml aprotinin, 10 mM phenylmethylsulfonyl fluoride). PKC activity was determined by measuring the transfer of 32P-labeled phosphate to a biotinylated peptide substrate (AAKIQASFGRHMARKK) that is specific for PKC activity using the Signa TECT™ PKC Assay System (Promega). In some experiments, the cells were treated with calphostin C (2 μM) (Biomol Research Laboratories, Plymouth, PA), a potent and selective inhibitor of PKC (53). Apoptotic cells were then detected by TUNEL analysis and counted as described above.

**RESULTS**

**Expression of BMP Signaling Effectors**—We first examined the expression of BMP signaling effectors in OHS4 and SaOS2 cells. Fig. 1 shows that the human OHS4 and SaOS2 cell lines express the full repertoire of Smad proteins (Smad1, -4, -5, and -8) (Fig. 1A) as well as BMP-RIA and BMP-RII mRNA, as assessed by RT-PCR analysis (Fig. 1B). The mRNA levels for BMP-RIA, BMP-RII, and Smads levels did not differ in OHS4 and SaOS2 cells. ALK2 was undetectable in OHS4 cells and was only barely detectable in SaOS2 cells (data not shown). Notably, BMP-RIB transcripts were detected in SaOS2 cells but not in OHS4 cells (Fig. 1B). Western blot analysis showed that, in contrast to SaOS2 cells, BMP-RIB protein levels were undetectable in OHS4 cells, confirming the lack of detectable BMP-RIB transcripts in these cells (Fig. 1C). These data show that BMPRIA and BMP-RII and Smad molecules that are effectors of BMP signaling are expressed in both SaOS2 and OHS4 cell lines, whereas OHS4 cells do not express detectable BMP-RIB.

**BMP-2 Promotes Osteoblast Gene Expression in Both OHS4 and SaOS2 Cells**—To investigate whether the expression of
BMP-RIB correlates with the effect of BMP-2 on osteoblast differentiation genes, OHS4 and SaOS2 cell lines were treated with BMP-2, and osteoblast markers induced by BMP-2 (7–13) were determined. As shown in Fig. 2A, BMP-2 dose-dependently increased ALP activity in OHS4 and SaOS2 cells with a similar dose-ranging effect. In addition, BMP-2 for 24 h increased ALP, CBFA1/RUNX2, and N-cadherin mRNA levels in both OHS4 and SaOS2 cells (Fig. 2B). Western blot analysis confirmed that BMP-2 treatment for 48 h increased N-cadherin and Cbfa1 protein levels in the two cell lines (Fig. 2C). These results suggest that BMP-2 promotes osteoblast marker genes independently of the level of expression of BMP-RIB in human osteoblastic cells.

Apoptosis Induced by BMP-2 Involves BMP-RIB and PKC Signaling—We therefore investigated whether BMP-2-induced apoptosis requires the expression of BMP-RIB in human osteoblastic cells. As shown in Fig. 3A, treatment with BMP-2 (12–125 ng/ml) for 24 h increased the number of TUNEL-positive apoptotic cells by up to 3-fold in serum-deprived SaOS2 cells. In contrast, BMP-2 had no effect on OHS4 cells. In these cells, etoposide used as a positive control induced apoptosis, confirming the validity of the TUNEL assay for detection of apoptosis in these cells (Fig. 3A). This suggests that OSH4 cells that lack BMP-RIB expression are unresponsive to BMP-2 in terms of induction of apoptosis. To confirm this finding, we identified the caspases that may be activated by BMP-RIB signaling, confirming the validity of the TUNEL assay for detection of apoptosis in these cells (Fig. 3A). This suggests that OSH4 cells that lack BMP-RIB expression are unresponsive to BMP-2 in terms of induction of apoptosis. To confirm this finding, we identified the caspases that may be activated by BMP-RIB signaling. Serum-deprived SaOS2 and OHS4 cells were treated with optimal dosages of BMP-2, and the activity of initiator caspases-2, -8, -9 and effector caspases-3, -6, and -7 was determined. As shown in Fig. 3B, treatment with BMP-2 (50 ng/ml) had no effect on caspase-2 or caspase-8 activity in OHS4 or SaOS2 cells. BMP-2 increased caspase-9 activity and effector caspases-3, -6, and -7 activity in SaOS2 cells but not in OHS4 cells (Fig. 3C). These results indicate that the pro-apoptotic effect of BMP-2 in SaOS2-responsive cells involves caspase-9 and effector caspases-3, -6, and -7, and confirm that OHS4 cells lacking BMP-RIB do not respond to BMP-2 in terms of caspase activity.

Our previous data showed that BMP-2 activates PKC in normal human osteoblasts (26). To test the hypothesis that BMP-RIB mediates the pro-apoptotic effect of BMP-2 via PKC activation, serum-deprived OHS4 and SaOS2 cells were treated with an optimal dose of BMP-2 (50 ng/ml) or its solvent, and PKC activity was determined. As shown in Fig. 4A, treatment with BMP-2 had no effect on PKC activity in OHS4 cells. In contrast, BMP-2 increased PKC activity at 5–10 min in SaOS2 cells, in line with our finding in human primary osteoblasts (26). To analyze whether the BMP-2-induced apoptosis mediated by BMP-RIB involves PKC signaling, serum-deprived SaOS2 and OHS4 cells were treated with BMP-2 in the presence of calphostin C, a selective inhibitor of PKC activity, and apoptosis was determined by TUNEL analysis. As shown in Fig. 4B, calphostin C (2 μM) completely suppressed apoptosis induced by BMP-2 (50 ng/ml) in SaOS2 cells. In contrast, calphostin C had no significant effect in OHS4 cells lacking BMP-RIB expression. These results indicate that the induction of apoptosis by BMP-2 in SaOS2 cells requires PKC signaling pathway downstream of BMP-RIB.

Apoptosis Induced by BMP-2 Overexpression Requires BMP-RIB—We then analyzed whether endogenous BMP-2 requires BMP-RIB expression to activate apoptosis in osteoblastic cells. To induce BMP-2 overexpression, OHS4 and SaOS2 cells were transfected with a BMP-2 cDNA vector. Validation of BMP-2 overexpression in transfected cells was determined by the expression of osteoblast marker genes. Western blot analysis showed that the two cell lines transiently transfected with the BMP-2 cDNA vector overexpressed the BMP-2 protein (Fig. 5).
BMP-RIB Mediates BMP-2-induced Osteoblast Apoptosis

5A). BMP-2 expression was increased by about 20% after 72 h of transfection, which corresponds to the efficiency of transfection (15–20%) determined by β-galactosidase activity in transfected cells. Transient overexpression of BMP-2 increased Cbfa1 protein levels by 30–50% in both OHS4 and SaOS2 cells, which is consistent with BMP-2 overexpression in these cells (Fig. 5A). These results indicate that endogenous BMP-2 promoted Cbfa1 expression, independently of the expression of BMP-RIB. We then examined the effect of endogenous BMP-2 on apoptosis. Transient overexpression of BMP-2 in SaOS2 cells increased apoptosis, as shown by the increased number of TUNEL-positive cells (Fig. 5B). In contrast, BMP-2 overexpression had no effect on apoptosis in OHS4 cells (Fig. 5B). These data, together with our finding that exogenous BMP-2 increased CBFA1/RUNX2 expression in both cell lines (Fig. 2), suggest that the expression of BMP-RIB is not an absolute requirement for the promotion of CBFA1/RUNX2 by BMP-2 in these human osteoblastic cells, whereas osteoblast apoptosis promoted by endogenous BMP-2 requires BMP-RIB expression.

A Dominant Negative BMP-RIB Suppresses Caspase Activity Induced by BMP-2—To examine further the role of BMP-RIB in BMP-2-induced apoptosis in osteoblastic cells, we established transfectants with a mutated BMP-RIB used as a dominant negative vector (40). As a positive control we used a cDNA expressing wild-type BMP-RIB. The efficiency of transfection was validated by determination of β-galactosidase activity after 72 h of transfection. Apoptosis was determined by the activation of caspase-9 and effector caspases-3, -6, and -7 which are involved in cell death induced by BMP-2 in these cells (see Fig. 3). As expected, treatment with BMP-2 for 24 h increased caspase-9 and caspases-3, -6, and -7 levels in SaOS2 cells transfected with the empty vector (Fig. 6A). Transfection with wtBMP-RIB further increased caspase-9 and caspases-3, -6, and -7 activity in response to BMP-2 in SaOS2 cells (Fig. 6A). This indicates that overexpression of BMP-RIB promotes BMP-2-induced apoptosis in SaOS2 cells. Consistently, transient transfection with DN-BMP-RIB blocked the BMP-2-induced activation of caspase-9 and caspase-3, -6, and -7 in SaOS2 cells (Fig. 6A). These results further indicate that BMP-RIB is essential for osteoblast apoptosis induced by BMP-2.

Forced Expression of BMP-RIB Rescues the Response to BMP-2 in OHS4 Cells—To document further the implication of BMP-RIB in BMP-2-induced apoptosis in osteoblastic cells, we determined whether expression of BMP-RIB in OHS4 cells may restore the effect of BMP-2 on caspase activity. As expected, OHS4 cells transfected with the empty vector did not respond to BMP-2 in terms of caspase-9 and caspases-3, -6, and -7 activity (Fig. 6B). Forced expression of wtBMP-RIB restored the BMP-2-induced activation of caspase-9 and caspases-3, -6, and -7 in OHS4 cells (Fig. 6B). To confirm further the role of BMP-RIB, OHS4 cells were transiently transfected with a constitutively active BMP-RIB. Transfection with caBMP-RIB increased basal levels of caspase-9 and caspases-3, -6, and -7 activity compared with the empty vector (Fig. 6B). Treatment with BMP-2 of OHS4 cells transfected with caBMP-RIB had no effect on caspase-9 and -3, -6, and -7 because caBMP-RIB precludes the response to BMP-2 stimulation (40). The finding that forced expression of wtBMP-RIB or a constitutively active form of BMP-RIB can restore the caspase response in OHS4 cells supports an essential role for BMP-RIB in the pro-apoptotic effect of BMP-2 in osteoblastic cells.

To confirm the role of BMP-RIB in normal osteoblastic cells, we used MC3T3-E1 osteoblastic cells that exhibit phenotypic characteristics of differentiating osteoblasts (47). BMP-2 (50 ng/ml) markedly enhanced caspases-3, -6, and -7 activity in MC3T3-E1 cells transfected with the empty vector (Fig. 6C). Transfection with wtBMP-RIB further increased caspases-3, -6, and -7 activity in response to BMP-2 in MC3T3-E1 cells (Fig. 6C). Furthermore, DN-BMP-RIB partially blocked the BMP-2-induced activation of caspases-3, -6, and -7 in MC3T3-E1 cells (Fig. 6C). The amplitude of effects of wtBMP-RIB and DN-BMP-RIB in these experiments (about 20%) corresponds to the efficiency of transfection (15–20%) determined by β-galactosidase activity in MC3T3-E1-transfected cells. These results in mouse osteoblastic MC3T3-E1 cells confirm the results in human SaOS2 cells indicating that BMP-RIB is involved in effector caspase activation induced by BMP-2 in osteoblasts.

Inhibition of Apoptosis Does Not Impair BMP-2-induced Promotion of Osteoblast Differentiation—Osteoblast apoptosis induced by BMP-2 and mediated by BMP-RIB may be secondary to promotion of osteoblast differentiation or may occur independently of cell differentiation induced by BMP-2. To clarify this point, SaOS2 cells were treated with Z-VAD-fmk, a broad spectrum caspases inhibitor (48), and cell number and osteoblast differentiation gene expression promoted by BMP-2 were...
In parallel experiments, we found that Z-VAD-fmk completely inhibits apoptosis induced by BMP-2 in osteoblastic cells, as evidenced by TUNEL staining (data not shown). Treatment of BMP-2-stimulated SaOS2 cells with Z-VAD-fmk for 72 h resulted in a 20% increase in cell number, reflecting the inhibitory effect of this broad caspase inhibitor on BMP-2-induced apoptosis (Fig. 7A). Notably, inhibition of caspases with Z-VAD-fmk for 72 h did not significantly reduce the BMP-2-induced stimulation of ALP activity in SaOS2 cells (Fig. 7A). Furthermore, RT-PCR analysis showed that Z-VAD-fmk did not block the increased expression of ALP or CBFA1/Runx2 induced by BMP-2 in SaOS2 cells (Fig. 7B). These data show that inhibition of effector caspases does not impair the promotion of osteoblast genes by BMP-2 in SaOS2 cells, indicating that BMP-2 signals osteoblast apoptosis independently of promotion of differentiation in these cells.

We also investigated whether inhibition of BMP-2-induced osteoblast apoptosis may alter matrix mineralization. To this goal, mouse osteoblastic MC3T3-E1 cells cultured in mineralizing conditions were treated with BMP-2 in the presence of the broad caspase inhibitor Z-VAD-fmk. Control MC3T3-E1 cells showed a marked mineralization of the matrix after 14 days of culture in mineralization medium, as assessed by von Kossa staining (Fig. 8A) and alizarin red staining (data not shown). Interestingly, continuous treatment with Z-VAD-fmk slightly increased matrix mineralization as shown by von Kossa staining (Fig. 8A). Quantification of calcium levels in the matrix confirmed that inhibition of caspases with Z-VAD-fmk significantly increased matrix mineralization by about 10% in MC3T3-E1 cultures (Fig. 8, A and B), indicating that inhibition of effector caspases results in increased matrix mineralization. Treatment with BMP-2 alone slightly decreased matrix mineralization and calcium deposition in MC3T3-E1 cell cultures after 14 days of mineralization (Fig. 8B), in line with a recent report (50) showing that BMP-2 slightly inhibits mineral deposition in MC3T3-E1 cultures. This inhibitory effect on matrix mineralization did not result from increased apoptosis induced by BMP-2 because inhibition of effector caspases with Z-VAD-fmk did not reverse the BMP-2-induced inhibition of calcium deposition (Fig. 8B). These results indicate that BMP-2 may control matrix mineralization in mouse MC3T3-E1 osteoblastic cells independently of osteoblast apoptosis.

**DISCUSSION**

Our results indicate that BMP-RIB is essential for BMP-2-induced apoptosis in osteoblastic cells and that BMP-RIB-mediated activation of apoptosis occurs independently of BMP-2-induced osteoblast differentiation. Type I BMP receptors are...
expressed in osteoblastic cells and are believed to mediate the activation of osteoblast differentiation by BMP-2 (3, 27). However, the promoting effect of BMP-2 may vary with the expression of BMP-RI which is distinctly expressed in osteoblastic cell lines (3). We took advantage of the distinct expression of BMP-RI in OHS4 and SaOs2 human osteoblastic cells used as a model to determine the role of BMP-RI in osteoblast differentiation and apoptosis promoted by BMP-2. Despite undetectable BMP-RI expression in OHS4 cells, BMP-2 increased the expression of ALP, Cbfal/Runx2, and N-cadherin which are BMP-responsive genes in human osteoblasts (11–13). Furthermore, overexpression of BMP-2 increased Cbfal/Runx2 expression similarly in the two cell lines. Thus, promotion of osteoblast marker genes by exogenous or endogenous BMP-2 is not entirely dependent on BMP-RI expression in human osteoblastic cells. These findings may appear in contrast with the previous finding that BMP-RI is required for the induction of osteoblast markers by BMP-2 in murine 2T3 calvaria cells (30) or for the differentiation of immature pre-osteoblasts in vivo (35). The apparent difference in BMP-RI requirement for promotion of differentiation by BMP-2 may be related to the differences in cell lines. Recent studies indicate, for example in MC3T3 calvaria cells (30), both BMP-RIA and BMP-RIB mediate osteoblast differentiation by BMP-2 in 3T3-F442A preadipocytes (31). Because BMP-RIA is expressed in OHS4 cells, this receptor may contribute to BMP-2-induced promotion of osteoblast differentiation in these cells.

The important role of BMP-RI in osteoblast differentiation by BMP-2 does not rule out the possibility that this receptor may control apoptosis. Although BMP-RIA signaling has been implicated previously in the regulation of embryonic programmed cell death during development (39, 54, 55), it remains unknown whether BMP-2 may promote apoptosis through a selective type I BMP-R in osteoblasts. Our present results indicate that activation of BMP-RIA plays a major role in the regulation of BMP-2-induced cell death in osteoblasts. This is supported by our finding that BMP-2 induced apoptosis in SaOs2 cells but not in OHS4 cells that do not express detectable BMP-RIA. Also, we documented that overexpression of BMP-2 increased caspases activity and DNA fragmentation in SaOs2 cells but not in OHS4 cells lacking BMP-RIA. Furthermore, BMP-2 was unable to induce PKC-driven apoptosis in OHS4 cells, whereas calphostin C, a potent inhibitor of PKC activity, abolished BMP-2-induced apoptosis. These results are consistent with a model in which BMP-RIA signals through PKC to control apoptosis in osteoblastic cells. Our finding that wtBMP-RIA enhanced the BMP-2-induced effector caspase activity and that a DN-BMP-RIA reduced the activity of effector caspases induced by BMP-2 in SaOs2 cells further indicates that BMP-RIA is essential for the induction of apoptosis by BMP-2 in osteoblasts. This is also supported by our finding that forced expression of BMP-RIA or transfection with caBMP-RIA restored effector caspase activity in OHS4 cells. The essential role of BMP-RIA in effector caspase activation by BMP-2 is not restricted to human osteosarcoma cell lines because we found similar results, albeit to a lesser magnitude, in mouse MC3T3-E1 osteoblastic cells. These results indicate that the apoptotic pathway mediated by BMP-2 in postnatal osteoblasts is triggered selectively through BMP-RIA in osteoblasts (36, 37). One hypothesis is that osteoblast apoptosis may occur secondary to the increased differentiation process induced by BMP-2. Alternatively, BMP-2-induced apoptosis may occur independently of its early effect on osteoblast differentiation. An interesting point is therefore to determine whether the induction of apoptosis by BMP-2 through BMP-RIA signaling is associated with change in osteoblast differentiation or is independent of it. Our results support the second alternative. We show that inhibition of effector caspases did not impair the induction of osteoblast differentiation genes by BMP-2 in SaOs2 cells. Consistent with this finding, BMP-2 increased osteoblast differentiation but not apoptosis in OHS4 cells lacking BMP-RIA. Thus, the induction of apoptosis by BMP-2 through BMP-RIA signaling may occur independently of osteoblast differentiation. These results suggest a dual role of BMP-RIA and BMP-RIB in osteoblast differentiation and a selective role of BMP-RIB in osteoblast apoptosis mediated by...
BMP-2. Given that apoptosis is essential for the control of the osteoblast lifespan (37, 38), our results point to a selective role of BMP-Rs in the functional regulation of osteoblast fate by BMP-2.

Other important points relevant to the functional regulation of osteoblasts and bone formation by BMP-2 are to determine whether inhibition of apoptosis may result in increased osteogenesis in vitro and whether induction of effector caspases by BMP-2 may result in changes in matrix mineralization in osteoblast cultures. To test these hypotheses, we used mouse osteoblastic MC3T3-E1 cells that are relatively differentiated osteoblasts and show osteogenic properties in vitro (47). We found that broad inhibition of effector caspases increased matrix mineralization in MC3T3-E1 cells. This finding suggests that inhibition of caspases in osteoblasts may contribute in the long term to increase osteogenesis. This result is consistent with the recent observations (37, 38) that anabolic agents can promote bone formation in vivo in part through inhibition of osteoblast apoptosis. Our finding that BMP-2 did not increase but rather decreased calcium deposition after 14 days of treatment is in line with a recent report (50) showing that BMP-2 slightly inhibits matrix mineralization in MC3T3-E1 cells. The negative effect of BMP-2 on matrix mineralization in MC3T3-E1 osteoblast cells does not appear to result from increased cell death because general inhibition of caspases did not reverse the effect of BMP-2 on matrix mineralization. Consistently, it was found recently (50, 56) that inhibition of mineralization by BMP-2 in MC3T3-E1 cells results from post-transcriptional inhibition of collagen accumulation by BMP-2. These results indicate that the control of matrix mineralization by BMP-2 occurs independently of the effector caspases activity induced by BMP-2 in MC3T3-E1 differentiating osteoblasts.

Taken together, our results show that BMP-RIP is essential for BMP-2-induced apoptosis in osteoblastic cells. Furthermore, our results strongly suggest that the control of osteoblast apoptosis by BMP-2 occurs independently of BMP-2-induced osteoblast differentiation genes, which provides additional insights into the dual mechanism of BMP-2 action on osteoblast fate. Apoptosis is known to be essential for the elimination of osteoblasts during skeletal development and remodeling, and this phenomenon controls osteoblast lifespan and thereby bone formation (37, 38). Our results suggest that, besides its prominent stimulatory effect on osteoblast differentiation, the pro-apoptotic effect of BMP-2-mediated by BMP-RIP signaling may control the number of differentiated osteoblasts, independently of its early effects on osteoblast gene expression and differentiation.

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