Signal Transductions Induced by Bone Morphogenetic Protein-2 and Transforming Growth Factor-β in Normal Human Osteoblastic Cells*

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Transforming growth factor β (TGF-β) activates Ras/MAPK signaling in many cell types. Because TGF-β and BMP-2 exert similar effects, we examined if this signaling is stimulated by both factors and analyzed the relationship between this signaling and the Smads in osteoblasts. BMP-2 and TGF-β stimulated Ras, MAPK, and AP-1 activities. The DNA binding activities of c-Fos, FosB/Ap1B, Fra-1, Fra-2, and JunB were up-regulated whereas JunD activity was decreased. c-Fos, FosB/ΔFosB, and JunB were associated with Smad4. The stimulation of AP-1 by BMP-2 and TGF-β was dependent on Smad signaling, and anti-Smad4 antibody interfered with AP-1 activity. Thus, BMP-2 and TGF-β activate both Ras/MAPK/AP-1 and Smad signaling in osteoblasts with Smads modulating AP-1 activity. To determine the roles of MAPK in BMP-2 and TGF-β function, we analyzed the effect of ERK and p38 inhibitors on the regulation of bone matrix protein expression and JunB and JunD levels by these two factors. ERK and p38 mediated TGF-β suppression of osteocalcin and JunD as well as stimulation of JunB. p38 was essential in BMP-2 up-regulation of type I collagen, fibronectin, osteopontin, osteocalcin, and alkaline phosphatase activity whereas ERK mediated BMP-2 stimulation of fibronectin and osteopontin. Thus, ERK and p38 differentially mediate TGF-β and BMP-2 function in osteoblasts.

Bone morphogenetic protein (BMP)-2 and transforming growth factor (TGF)-β, which are members of the TGF-β superfamily and share 32–37% sequence homology, have profound effects on osteoblast activity (1–6). Both in vivo and in vitro bone formation potential of BMP-2 has been well documented. When implanted intramuscularly or subcutaneously, BMP-2 induces ectopic bone formation with complementary marrow cavity and marrow cell constituents (1–3). In vitro, BMP-2 increases the colony formation of normal human bone marrow stromal preosteoblasts and induces their differentiation into cells with mature osteoblastic phenotype (6–8). TGF-β, one of the major growth factors present in bone matrix that functions as a putative coupling factor between bone formation and bone resorption, also has potent osteoinductive capability in vivo (4, 5, 9, 10). Although conflicting and opposing results have been reported on osteoblast proliferation and differentiation depending on the osteoblast model used, the most noticeable effects of TGF-β on human osteoblasts are stimulation of proliferation and bone matrix protein deposition, although it depresses the synthesis of osteocalcin (4, 5, 11–14). In addition to the effects on osteoblast growth and differentiation, both BMP-2 and TGF-β can induce osteoblast chemotaxis, which is essential for bone formation to occur (4, 15–19).

Recently, Smad signaling has been well characterized to mediate TGF-β and BMP-2 activity in a variety of cells, including osteoblasts (20–25). Upon binding to their respective receptors, pathway-specific Smad proteins (Smad1 and Smad5 for BMP-2; Smad2 and Smad3 for TGF-β) are activated and form complexes with Smad4. These complexes are subsequently transported into the nucleus to exert their gene regulation directly or indirectly. Although Smad signal transduction pathways appear to be the major mediators for TGF-β and BMP-2, other signaling molecules, such as Ras and MAPK, are also activated by these two factors in various cell systems (26, 27). Furthermore, the induction of collagen and fibronectin and the suppression of cell proliferation by TGF-β are maintained in Smad4-null cancer cells and fibroblasts (28–30). These combined data suggest that one or more Smad-independent signaling mechanisms also mediate TGF-β and BMP-2 activity. In osteoblasts, both BMP-2 and TGF-β stimulate the expression of c-Fos, an AP-1 component (31–33). Because Ras is the upstream effector of Fos and MAPK, and AP-1-responsive element is present in the promoters of major bone matrix proteins such as type I collagen, osteocalcin, osteopontin, and fibronectin, the induction of Ras/MAPK/AP-1 appears to be an important signal transduction pathway in mediating part of the effects of BMP-2 and TGF-β in osteoblasts. Therefore, we analyzed the effect of BMP-2 and TGF-β on the activity of Ras, MAPK, and AP-1 in normal human osteoblastic cells (HOB). It has been shown that the interaction between Smad proteins and AP-1 components are critical in TGF-β function (34–36). No information, however, is available as to whether this interaction is also essential in BMP-2 function. Therefore, the relationship between AP-1 and the Smad signaling induced by TGF-β and BMP-2 in osteoblasts and the roles of ERK and p38 MAPK in the regulation of osteoblast function by TGF-β and BMP-2 were investigated.
**EXPERIMENTAL PROCEDURES**

**Materials—**TGF-β2 and BMP-2 were generously provided by Dr. Nico C. Cerletti (Novartis Pharma AG, Basel, Switzerland) and Genetics Institute (Cambridge, MA), respectively. All chemicals for SDS-PAGE and protein assays were from Bio-Rad (Richmond, CA). pAP1-Luc plasmid, which is a cis-reporter plasmid containing a seven tandem-AP1 enhancer element (TGACTAA), and pC-MEKK were from Stratagene (La Jolla, CA). Rat osteocalcin promoter (−637/−32) conjugated to Luciferase reporter cDNA (pOC-Luc) was kindly provided by Dr. Dwight A. Towler (Washington University, St. Louis, MO). DEAEdextran, consensus oligonucleotide for NFκB (5′-AGTTAGGGACTCTCCGAGCC-3′) and a β-Galactosidase Enzyme Assay System with Reporter Lysis Buffer kit were from Promega Co. (Madison, WI). LipofectAmine and pcDNA3 plasmids were from Invitrogen (Rockville, MD). Promega 486 was from Research Genetics (Huntsville, AL). 

**Cell Culture—**Normal human osteoblastic cells (HOB) were isolated as described previously (37). Briefly, trabecular bone chips were scraped off of ribs and digested with collagenase for 2 h. The remaining bone chips were cultured for 4–6 weeks in Dulbecco’s modified Eagle’s medium nutrient mixture F-12 Ham’s containing 10% heat-inactivated fetal bovine serum (HIFBS). HOB outgrew from bone chips were subcultured into α-MEM medium containing 10% HIFBS. The first and second passaged cells were used for assays. The murine osteoblast cell line MC3T3-E1, which exhibits similar property as HOB, was employed for transfection experiment, because HOB was prepared as described (38). Briefly, cells were lysed in ice-cold buffer consisting of 10 mM Hepes-KOH, pH 7.9, 1.5 mM MgCl2, 10 mM KCl, 0.5 mM dithiothreitol (DTT), 0.5 mM PMSF, and 0.6% Nonidet P-40 for 10 min. After microcentrifugation, pellets were extracted with high salt buffer (20 mM Hepes-KOH, pH 7.9, 1.2 mM MgCl2, 420 mM NaCl, 25 mM glycerol, 0.5 mM DTT, 0.5 mM PMSF, 10 µg/ml each of leupeptin and pepstatin, and 25 µg/ml aprotenin) to obtain nuclear extracts. Protein concentration in the nuclear extracts was measured using the Bio-Rad protein assay kit. For electrophoretic mobility shift assay (EMSA), only the first and second passaged cells were used for assays. The incubation period, cells were treated with vehicle (Control), BMP-2 (100 ng/ml), pAP1-Luc, whole cell lysates in MAPK assay buffer (50 mM Tris-Cl, pH 7.4, 150 mM NaCl, 5 mM EDTA, 1% Nonidet P-40, 150 µg/ml leupeptin, protease inhibitor mixture (Sigma) and tyrosine phosphatase inhibitor (100 µg/ml poly(dI-dC)) at room temperature for 20 min as described previously (40). Assays were terminated by the addition of 1 µl of loading buffer. Samples were subjected to electrophoresis using 4% Tris borate, pH 8.3, 0.3% agarose gel (3% Tris borate, pH 8.3, and 0.6 mM EDTA) at 100 V for 2.5 h at room temperature. Gels were dried, and autoradiography was performed. For competitive EMSA or antibody supershift assays, 100-fold of unlabeled double-stranded oligonucleotide (1.75 pmol) or the indicated antibody (1 µg) was incubated with nuclear extracts for 30 min before the addition of radioactive probe.

**Western Blot Analysis—**Nuclear extracts, obtained as described above, or the whole cell lysates in MAPK assay buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 5 mM EDTA, 1% Nonidet P-40, 150 µg/ml NaF, protease inhibitor mixture (Sigma) and tyrosine phosphatase inhibitor (100 µg/ml poly(dI-dC)) at room temperature for 20 min as described previously (40). Assays were terminated by the addition of 1 µl of loading buffer. Samples were subjected to electrophoresis using 4% Tris borate, pH 8.3, and 0.6 mM EDTA at 100 V for 2.5 h at room temperature. Gels were dried, and autoradiography was performed. For competitive EMSA or antibody supershift assays, 100-fold of unlabeled double-stranded oligonucleotide (1.75 pmol) or the indicated antibody (1 µg) was incubated with nuclear extracts for 30 min before the addition of radioactive probe.

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**Nuclear Extract Preparation and Electrophoresis Mobility Shift Assay—**Nuclear extracts of HOB, which were previously described with BMP-2, TGF-β, or vehicle for the indicated period of time, were prepared as described (38). Briefly, cells were lysed in ice-cold buffer consisting of 10 mM Hepes-KOH, pH 7.9, 1.5 mM MgCl2, 10 mM KCl, 0.5 mM dithiothreitol (DTT), 0.5 mM PMSF, and 0.6% Nonidet P-40 for 10
RESULTS

**BMP-2 and TGF-β Stimulated Ras Activity in HOB—** Exposure of HOB to BMP-2 for 5 and 10 min resulted in an increased Ras activity to 5- and 12-fold of the control level, respectively (Fig. 1). TGF-β also activated Ras activity to 4-fold of the control level after 5-min exposure, which tapered off after 10 min (Fig. 1).

**BMP-2 and TGF-β Up-regulated MAPK Activity**—Because Ras can activate ERK of the MAPK superfamily, we next examined the effect of BMP-2 and TGF-β on ERK activity. BMP-2 stimulated ERK activity 5-fold after 1 h of incubation as demonstrated by the increased phosphorylated ERK (p-ERK) level relative to the total ERK level (pan-ERK) (Fig. 2A). The stimulation of ERK activity by BMP-2, however, was nearly over after 6 h (Fig. 2A). In contrast, TGF-β only marginally stimulated ERK activity after 1 h of incubation, which became remarkable after 6 h (5-fold, Fig. 2B). Because JNK and p38 also belong to the MAPK superfamily (42–44), we analyzed the effect of BMP-2 and TGF-β on their activity. As shown in Fig. 3A, BMP-2 stimulated p38 and ERK activity, but not JNK activity, after 1-h incubation. Two hours after incubation with BMP-2, the stimulation of ERK persisted and JNK was activated, whereas p38 was no longer activated. TGF-β had little effect on ERK, JNK, and p38 activity after 1 h (Fig. 3B). Two hours after addition of TGF-β, JNK and p38, but not ERK, were activated (Fig. 3B). Thus, BMP-2 and TGF-β stimulated ERK, JNK, and p38 MAPK activities differently, depending on the length of treatment.

**BMP-2 and TGF-β Stimulated AP-1 Activity**—Because components in the AP-1 complex serve as substrates for MAPK (45, 46), we examined the effect of BMP-2 and TGF-β on AP-1 activity. Using a pAP1-Luc plasmid, which carries a seven-nucleotide than the control extracts (Fig. 5, left). This binding was AP-1-specific, because it was blocked only by the non-labeled AP-1, but not NFκB, consensus oligonucleotide (Fig. 5, left). Furthermore, no complex formation was detected in all the samples tested when a mutated AP-1 consensus oligonucleotide (AP-I_m) was used as the radiolabeled probe (Fig. 5, right).

**Identification of the Members of the AP-1 Superfamily Stimulated by BMP-2 and TGF-β**—It has been well documented that members of Fos/Jun and CREB/ATF2 families can bind to the AP-1-responsive element (47–49). We next examined the components in the AP-1 complex that were regulated by TGF-β and BMP-2. Incubation of nuclear extracts, which were obtained 6 h after exposure to TGF-β, with antibodies against FosB, Fra-2, and JunB led to retardation of the migration of AP-1-DNA complex in EMSA (Fig. 6A, bottom panel, arrows). Because the intensity of these supershifted bands was higher in TGF-β-stimulated samples than those in the control samples,
these results suggested that TGF-β up-regulated the activity of FosBΔFosB, Fra-2, and JunB. Although antibody to JunD also produced supershifted bands (Fig. 6A, top panel), no difference was detected in their intensity between the control and TGF-β-treated samples. Thus, TGF-β did not affect JunD activity after 6-h stimulation. Judging from the remaining AP-1-DNA band intensity after incubation with specific antibodies against c-Fos, c-Jun, and ATF-2, TGF-β had either very little or no effect on the activities of these AP-1 components 6 h after treatment (Fig. 6A, top panel).

When nuclear extracts were preincubated with anti-Fra-1 antibody before the addition of radiolabeled oligonucleotide probe, the remaining AP-1-DNA band intensity in the TGF-β-treated sample was reduced as compared with the sample in which no antibody was added (Fig. 6A, top panel, compare lanes 7 and 8 with lanes 1 and 2, respectively). Because anti-c-Fos and anti-ATF2 antibodies did not alter the AP-1-DNA band intensities in the TGF-β-treated samples under the same experimental condition (Fig. 6A), the reduction of the AP-1-DNA band intensity in TGF-β-treated sample by anti-Fra-1 antibody appeared to be specific. This suggested that Fra-1 was stimulated by TGF-β, because more Fra-1-Jun complexes were removed from this sample than the control. The absence of a supershifted band in the presence of anti-Fra-1 antibody despite a prolonged exposure to x-ray films (data not shown) may derive from the destabilization of the Fra-1-DNA complex by this antibody as a result of antibody competition with the oligonucleotide probe for Fra-1 or alteration of the conformation of Fra-1 by antibody.

Because AP-1 is composed of a large array of Jun-Jun or Fos-Jun dimers, each individual Fos-Jun member constitutes only a portion of this mixture, depending on the abundance of each dimer. Removal of a portion of these dimers by antibody targeting to a specific Fos-Jun member may not alter the remaining AP-1 band intensity greatly, although the change is significant. To further confirm the up-regulation of the activity of Fos family members by TGF-β, we employed an anti-pan-Fos antibody, which recognizes the common domain of the Fos family members and can interact with a vast number of Fos-Jun dimers, in EMSA. As shown in Fig. 6A, top panel, this antibody clearly supershifted the AP-1-DNA bands in the gel, leaving behind non-detectable AP-1-DNA bands in the original location in the short-exposure film. Furthermore, the intensity of the supershifted band was higher in TGF-β-treated sample than in control sample, confirming that TGF-β stimulated the DNA-binding activity of Fos members of the AP-1 family.

The effect of TGF-β on the activities of AP-1 components was also examined after 2-h exposure. Although TGF-β did not appear to have any effect on the total AP-1-DNA-binding activity at this time point, supershifted bands induced by anti-JunD antibody revealed that JunD activity was inhibited by TGF-β whereas the remaining AP-1-DNA band was more intense in TGF-β-treated samples (Fig. 6B). These data suggested that TGF-β inhibited JunD but stimulated the activities of at least some of the remaining AP-1 complexes. In contrast to the lack of effect on c-Fos activity observed after 6-h exposure described above, TGF-β up-regulated c-Fos activity after 2 h, because incubation of the nuclear extract with specific anti-c-Fos antibody reduced the AP-1 band intensity in the TGF-β lane to less than that of the control in EMSA (Fig. 6B). The activities of FosBΔFosB and JunB were also up-regulated by TGF-β at this time point whereas those of Fra-1, Fra-2, c-Jun, and ATF-2 were not altered (data not shown).

BMP-2 also differentially regulated the activity of the individual AP-1 component. Supershift EMSA indicated that FosBΔFosB and Fra-2 were stimulated by BMP-2 whereas JunD was inhibited after 6-h exposure to BMP-2 (Fig. 7). c-Fos, Fra-1, c-Jun, JunB, and ATF-2 were also significantly up-regulated, because their antibodies reduced or abolished the stimulation of AP-1 band by BMP-2 (Fig. 7). The supershifted bands obtained using pan-Fos antibody further confirmed the
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Fig. 6. TGF-β differentially modulates the binding activity of individual AP-1 components to AP-1 consensus oligonucleotide. Nuclear extracts (NE) obtained from cells treated with either vehicle (C) or TGF-β (T, 1 ng/ml) for 6 h (A) or 2 h (B) were preincubated with the indicated antibody (1 μg) for 30 min before the addition of radiolabeled AP-1 consensus oligonucleotide. Complexes formed were subjected to EMSA, and bands were visualized by autoradiography. The bottom panels in A were after prolonged exposure to show the supershifted bands more clearly (arrows). In the FosB/ΔFosB panel, the top arrow represents FosB and the bottom arrow indicates ΔFosB. The top panel in A was compiled from three gels, which were performed simultaneously and exposed to the x-ray films for the same length of time.

Fig. 7. BMP-2 differentially modulates the binding activity of individual AP-1 components to AP-1 consensus oligonucleotide. Nuclear extracts (NE) obtained from cells treated with either vehicle (C) or BMP-2 (B, 100 ng/ml) for 6 h were preincubated with the indicated antibody (1 μg) for 30 min before the addition of radiolabeled AP-1 consensus oligonucleotide. Complexes formed were subjected to EMSA and bands visualized by autoradiography. The bottom panel is after prolonged exposure to show the supershifted bands of FosB (upper arrow) and ΔFosB (lower arrow) more clearly.

Fig. 8. c-Fos, JunB, FosB, and ΔFosB are associated with Smad4 in HOB. Cells were stimulated with vehicle (C) or TGF-β (T) for 2 h (left), or vehicle (C) or BMP-2 (B) for 6 h (right). Nuclear extracts were subjected to immunoprecipitation with anti-Smad4 antibody followed by Western blotting and probed for c-Fos, JunB, and FosB/ΔFosB. Antibody against FosB/ΔFosB detects two bands with FosB in the upper band and ΔFosB in the lower band.

The importance of Smad proteins in the regulation of AP-1 activity was further demonstrated by using MC3T3-E1 osteoblastic cell line overexpressing dominant negative Smad3 (Smad3m), Smad4 (Smad4m), or Smad5 (Smad5m) or the pcDNA3 control vector. As shown in Fig. 9A, the fold stimulation of AP-1 activity by TGF-β in Smad3m and Smad4m cells was 2.0- and 1.7-fold, respectively, which was substantially less than the 3.2-fold obtained in pcDNA3 control cells. Similarly, BMP-2 stimulated AP-1 activity was decreased from 1.7- to 0.8- and 0.6-fold, respectively, in Smad5m and Smad4m cells (Fig. 9B). The role of Smad signaling in AP-1 activation was further confirmed by co-transfecting MC3T3-E1 cells with a pAP1-Luc plasmid and the expression vectors of MEKK and either the Smad3/Smad4 or Smad5/Smad4 pair. MEKK alone stimulated
AP-1 activity to 48.3-fold of the basal level whereas Smad3 and Smad4 together enhanced AP-1 activity 2-fold (Fig. 10, compare column 2 and 3 with column 1). The combination of MEKK, Smad3, and Smad4 enhanced AP-1 activity to 77-fold of the basal level, which is substantially higher than the sum of MEKK and Smad3/Smad4 stimulation (Fig. 10, compare column 4 with column 1). Similarly, Smad5 and Smad4 together stimulated AP-1 activity to 1.5-fold of the basal level (Fig. 10, compare column 5 with column 1) and the combination of Smad5, Smad4, and MEKK resulted in a 70-fold stimulation of AP-1 activity (Fig. 10, compare columns 6 with column 1). These data implicated that the Smad3/Smad4 and Smad5/Smad4 pairs not only could stimulate AP-1 activity they also synergistically enhanced the up-regulation of AP-1 by MEKK.

**ERK and p38 Differentially Mediated BMP-2 and TGF-β Effects on Osteoblast Function**—With the establishment that both BMP-2 and TGF-β stimulate MAPK activity in osteoblasts, we analyzed the roles of MAPK in mediating the effects of these two factors on these cells. Because the major function of osteoblasts is to deposit bone matrix proteins and mineralize the matrix, we employed PD98059 and SB208530 to determine if ERK and p38 mediate TGF-β and BMP-2 effects on the expression of bone matrix proteins important for matrix mineralization. It has been shown that TGF-β inhibited osteocalcin expression in various osteoblastic cells (51–53). Consistently, TGF-β inhibited the osteocalcin promoter activity in MC3T3-E1 osteoblastic cells, and this inhibition was prevented by PD98059 (Fig. 11A). In contrast, BMP-2 stimulated osteocalcin expression, which was found to be dependent on p38 but not ERK, because SB208530, not PD98059, abrogated this stimulation (Fig. 11B).

Both TGF-β and BMP-2 enhanced the levels of fibronectin, type I collagen, and osteopontin in HOB after a 3-day exposure (Fig. 12). Surprisingly, neither PD98059 nor SB208530 had any inhibitory effect on the up-regulation of these proteins by TGF-β (Fig. 12A), suggesting that neither ERK nor p38 mediate these TGF-β effects. The lack of effect of PD98059 and SB208530 was not due to their loss of activity, because both suppression of the stimulation of JunB and abrogated the down-regulation of JunD by TGF-β (Fig. 12A). p38 was found to play an important role in mediating BMP-2 up-regulation of fibronectin, type I collagen, and osteopontin, because SB208530 suppressed these stimulation (Fig. 12B). Similarly, ERK was essential in BMP-2 up-regulation of fibronectin and osteopontin, but not type I collagen, because PD98059 suppressed the stimulation of the former two proteins but not the latter one (Fig. 12B).
down- and up-regulated, respectively, after exposure to BMP-2 for 6 h (Fig. 7), their levels were not altered after a 3-day treatment by BMP-2 (Fig. 12B). This transient up-regulation of JunB by BMP-2 has also been reported at the mRNA level (54).

Alkaline phosphatase, which is a membrane-bound enzyme important for matrix mineralization, is known to be a target of BMP-2 (6). Therefore, we also examined the role of ERK and p38 in mediating BMP-2 up-regulation of this enzyme. TGF-β, which can either stimulate or inhibit alkaline phosphatase activity depending on the cell system used, was also analyzed. TGF-β had very little effect on the alkaline phosphatase activity in HOB whether in the presence or absence of the inhibitors (Fig. 13A). In contrast, BMP-2 stimulated alkaline phosphatase activity by more than 2-fold and this up-regulation was inhibited by SB203580 but not by PD98059 (Fig. 13B). Thus, p38, but not ERK, mediates BMP-2 up-regulation of alkaline phosphatase activity. In conclusion, ERK and p38 differentially mediate the regulation of bone matrix protein expression and alkaline phosphatase activity by TGF-β and BMP-2 in osteoblasts.

**DISCUSSION**

We have demonstrated that Ras/MAPK/AP-1 signal transduction is induced by both TGF-β and BMP-2 in osteoblasts in addition to Smad signaling. The majority of AP-1 components are stimulated to various degrees by these two growth factors whereas JunD is inhibited. We have also demonstrated that Smad signaling is essential in the AP-1 up-regulation by TGF-β and BMP-2. Although both TGF-β and BMP-2 stimulate Ras and all three members of MAPK (ERK, p38, and JNK), their time course profiles differ. The stimulation of Ras by BMP-2 is stronger and is maximal after 10 min whereas the stimulation by TGF-β is moderate and the maximum occurs after 5 min. The activation of ERK and p38 by BMP-2 occurs after 1 h of incubation whereas a robust response to TGF-β requires 6 and 2 h, respectively. The timelines of the regulation of some of the AP-1 components by these two growth factors also differ. The stimulation of c-Fos and the inhibition of JunD activity in the nuclei by TGF-β are only detectable after 2-h exposure whereas the effects of BMP-2 on these two AP-1 components persist after 6 h. In addition, ATF-2 is clearly activated by BMP-2, whereas its regulation by TGF-β is less obvious. Thus, TGF-β and BMP-2 exert a similar, but not identical, effect on the regulation of AP-1 family members, c-Jun activity is not altered by TGF-β, whereas JunD is inhibited. We have also demonstrated that the majority of AP-1 components by these two growth factors also differ. The stimulation of Ras by BMP-2 is stronger and is maximal after 10 min whereas the stimulation by TGF-β is moderate and the maximum occurs after 5 min.

**Ras/MAPK/AP-1 signal transduction in normal human osteoblast cells.** In addition, we have shown that ERK and p38 mediate differentially TGF-β and BMP-2 effects on osteoblast function. Both ERK and p38 mediate TGF-β stimulation of JunB and suppression of JunD and osteocalcin expression. In contrast, p38 plays an important role in BMP-2 up-regulation of the expression of type I collagen, fibronectin, osteopontin, osteocalcin, and alkaline phosphatase activity, and ERK is also essential in BMP-2 up-regulation of fibronectin and osteopontin.

The roles of AP-1 components in bone formation and osteoblast function have been amply demonstrated. Transgenic mice overexpressing c-Fos develop osteosarcoma (55) whereas those overexpressing Fra-1 or ΔFosB have increased bone formation, expression of bone matrix proteins and Cbfa1, and alkaline phosphatase activity (56, 57). Cbfa1 is an essential transcription factor, which regulates the expression of many important bone matrix proteins, including osteocalcin in osteoblasts (58, 59). Mice deficient in ATF-2 have a defect in endochondral ossification at epiphyseal plates (60). However, transgenic mice overexpressing c-Jun or FosB do not develop any skeletal pathology despite high expression in bone tissue (55).

Active bone-forming osteoblasts, but not bone lining cells or osteocytes, express high levels of c-Fos and c-Jun mRNAs as demonstrated by *in situ* hybridization (61). In osteoblast cultures, c-fos, c-jun, and junB mRNA are expressed at high levels during proliferative period whereas those of c-fos and junB are enhanced during differentiation phase (62). c-Fos and JunB also mediate either the mitogenic or the anti-mitogenic effect of TGF-β depending on the osteoblast systems used (33, 63). Although TGF-β regulates the activities of most of the AP-1 members, c-Jun activity is not altered by TGF-β. The lack of stimulation of c-Jun by TGF-β has also been reported earlier in HOB, in which TGF-β actually reduces c-Jun mRNA level (31). Although transgenic mice overexpressing ΔFosB have elevated...
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type I collagen expression (57), the up-regulation of α1(I) collagen expression induced by TGF-β and BMP-2 and the stimulation of parathyroid hormone/parathyroid hormone-related peptide receptor by TGF-β are inhibited in ROS17/2.8 osteosarcoma cells overexpressing ΔFosB (64). Both c-Fos and Fra-2 have been implicated to be important for osteoblast differentiation (32, 47). Furthermore, AP-1 complex composed of Fra-2 and JunD stimulates osteocalcin expression (47). It is of note that JunB mediates the inhibition of the myogenic differentiation of C2C12 cells by BMP-2 and TGF-β (66). Because osteoblasts and myocytes are derived from the same mesenchymal progenitor cells and BMP-2 stimulates the differentiation of C2C12 toward osteoblastic phenotype with the concomitant inhibition of myogeneses (24), JunB may play an important role in dictating the differentiation of the mesenchymal progenitor cells toward osteoblastic phenotype. These combined data suggest that c-Fos, Fra-1, Fra-2, ΔFosB, and JunB mediate, at least in part, the effects of TGF-β and BMP-2 on osteoblast differentiation and matrix protein expression and their in vivo bone formation activity.

The roles of Smad proteins in bone formation and osteoblast function have been well recognized. Smad1, which mediates BMP-2 effects, has been shown to induce osteoblast differentiation (68). Smad2 and Smad3, which are activated by TGF-β, are essential for the proper development of skeleton and craniofacial bones, respectively (69). Mice deficient in Smad3 suffer osteoporosis, abnormal ossification of the joints, and osteoarthritis (70) whereas heterozygotes of Smad2-deficient mice lack mandible (71). With the demonstration that Smad signaling is essential in the activation of AP-1 by BMP-2 and TGF-β (this report and Refs. 34–36), the importance of Smad signaling in bone formation is further affirmed. Although TGF-β and BMP-2 stimulate the activity of most of the AP-1 components, the most intriguing observation is their inhibition of JunD activity. JunD is constitutively expressed in osteoblasts, and its level declines only slightly during differentiation (62). Because JunD together with Fra-2 is essential for osteocalcin expression (47), the reduction of JunD level may explain the inhibition of osteocalcin expression by TGF-β. Despite the inhibition of JunD activity after a 6-h treatment, BMP-2 stimulates osteocalcin expression in osteoblasts, consistent with earlier reports (6, 72). Because no suppression of JunD level by BMP-2 is detected after a 3-day treatment (Fig. 12) and BMP-2 has been shown to stimulate osteocalcin expression via Cbfα1 in a Smad-dependent manner (73, 74), the transient decline in JunD may not be sufficient to counter the stimulatory effect of Cbfα1 on osteocalcin expression.

Although the close relationship between Smad and Ras/MAPK/AP-1 is confirmed in osteoblasts, it is of note that osteoblasts expressing a dominant negative form of Smad3 or Smad4 only lessen the effects of TGF-β on AP-1 activity whereas the dominant-negative Smad5 or Smad4 abrogates completely BMP-2 effects (Fig. 9). Recently, Piek et al. (67) demonstrated that the induction of c-fos mRNA by TGF-β is dependent on Smad3 but not Smad2 in studies utilizing embryonic fibroblasts derived from either Smad2- or Smad3-deficient mice. The disparity in Smad2 and Smad3 function is also shown in the repression of Cbfα1 by TGF-β, in which Smad3 but not Smad2 mediates this effect (53). Thus, there is a differential regulation of AP-1 activity by various Smad proteins. Two mechanisms are known to mediate the regulation of AP-1 activity by Smad proteins. First, Smad proteins can directly regulate AP-1 activity by protein-protein interaction with the components of AP-1 family members as shown in this report and by others (34–36). Second, Smad proteins can regulate the transcription of AP-1 components. For example, the synthesis of JunB is stimulated by TGF-β and BMP-2 via Smad binding element (CAGACA) in the JunB promoter (65).

Although BMP-2 stimulates the activities of ERK and p38, these two MAPKs play different roles in BMP-2 regulation of osteoblast function. p38 mediates BMP-2 up-regulation of fibronectin, type I collagen, osteopontin, osteocalcin, and alkaline phosphatase activity whereas ERK is important only for the up-regulation of fibronectin and osteopontin. TGF-β also stimulates ERK and p38. However, these two MAPKs only mediate TGF-β down-regulation of osteocalcin but not up-regulation of fibronectin, type I collagen, and osteopontin. It is of note that p38 mediates both BMP-2 up-regulation and TGF-β down-regulation of osteocalcin. One of the possible explanations for these differential roles of ERK and p38 played in TGF-β and BMP-2 regulation of bone matrix protein expression could reside in the distinct Smad proteins activated by these two factors. Although pathway-specific Smad proteins exhibit mostly similar function, distinctive activity reserved for individual Smad protein has been reported with increasing frequency as described above. Because Smad can modulate AP-1 activity, the activity of specific AP-1 member activated by ERK or p38 may be modulated differentially by each Smad protein, leading to a disparate bone matrix protein expression. The temporal difference in the activation of each member of MAPK and AP-1 family by TGF-β and BMP-2 may also contribute to the differential roles of ERK and p38 in mediating TGF-β and BMP-2 regulation of matrix protein expression.

In conclusion, TGF-β and BMP-2 activate not only the Smad signaling but also the Ras/MAPK/AP-1 pathway. These two signaling activations converge at the AP-1 level with Smad proteins regulating AP-1 activity. Members of the AP-1 and MAPK family are important mediators in BMP-2 and TGF-β regulation of gene expression in osteoblasts. The net effect of these two factors on gene expression depends on the intricate balance of these two signal transduction pathways.

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