 Requirement of BMP-2-induced Phosphatidylinositol 3-Kinase and Akt Serine/Threonine Kinase in Osteoblast Differentiation and Smad-dependent BMP-2 Gene Transcription*

Received for publication, May 22, 2002
Published, JBC Papers in Press, June 25, 2002, DOI 10.1074/jbc.M205053200

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The mechanism by which bone morphogenetic protein-2 (BMP-2) induces osteoblast differentiation is not precisely known. We investigated the involvement of the phosphatidylinositol (PI) 3-kinase/Akt signal transduction pathway in modulation of this process. BMP-2 stimulated PI 3-kinase activity in osteogenic cells. Inhibition of PI 3-kinase activity with the specific inhibitor Ly-294002 prevented BMP-2-induced alkaline phosphatase, an early marker of osteoblast differentiation. Expression of dominant-negative PI 3-kinase also abolished osteoblastic induction of alkaline phosphatase in response to BMP-2, confirming the involvement of this lipid kinase in this process. BMP-2 stimulated Akt serine/threonine kinase activity in a PI 3-kinase-dependent manner in osteoblast precursor cells. Inhibition of Akt activity by a dominant-negative mutant of Akt blocked BMP-2-induced osteoblastic alkaline phosphatase activity. BMP-2 stimulates its own expression during osteoblast differentiation. Expression of dominant-negative PI 3-kinase or dominant-negative Akt inhibited BMP-2-induced BMP-2 transcription. Because all the known biological activities of BMP-2 are mediated by transcription via BMP-specific Smad proteins, we investigated the involvement of PI 3-kinase in Smad-dependent BMP-2 transcription. Smad5 stimulated BMP-2 transcription independent of addition of the ligand. Dominant-negative PI 3-kinase or dominant-negative Akt inhibited Smad5-dependent transcription of BMP-2. Furthermore, dominant-negative Akt inhibited translocation of BMP-specific Smads into nucleus. Together these data provide the first evidence that activation of BMP receptor serine/threonine kinase stimulates the PI 3 kinase/Akt pathway and define a role for this signal transduction pathway in BMP-specific Smad function during osteoblast differentiation.

Bone morphogenetic proteins (BMPs), a group of polypeptides within the transforming growth factor (TGF)-β superfamily, were originally identified by their ability to induce endochondral bone formation in ectopic extraskeletal sites in vivo (1). BMPs stimulate differentiation of pluripotent mesenchymal cells into the osteogenic lineage and enhance the differentiated function of osteoblasts. Among many other BMPs, BMP-2 induces differentiation of preosteoblasts into mature osteoblasts by regulating signals that stimulate a specific transcriptional program required for bone formation (2, 3).

Similar to TGF-β, BMPs exert their effect via type I and type II transmembrane serine/threonine kinase receptors (4, 5). The type II receptor binds the ligand with high affinity. The type I receptors, Alk3 (BMPRIA) and Alk6 (BMPRIB) are mainly responsible to transduce the signal, although they have weak BMP binding properties (6). Binding of BMP to its receptors induces phosphorylation of the type I receptor in its GS domain and recruitment of receptor-specific Smads (4, 5, 7). Upon phosphorylation, Smad1, or one of its close homologs Smad5 and Smad8, heterodimerizes with the common Smad, Smad4. This complex translocates to the nucleus where it associates with transcriptional coactivators and acts as a transcription factor to regulate tissue or cell type-specific genes required for the divergent biological functions of BMPs (4, 5, 7). In osteogenic cells, BMP-specific Smads stimulate expression of Cbfa1, the only known osteoblast-specific transcription factor to induce genes required for mature osteoblast differentiation and maintenance (8, 9).

Differentiation in response to extracellular factors depends on the action of kinases including both tyrosine and serine/threonine kinases (10, 11). In the case of neuronal differentiation, the tyrosine kinase activity of nerve growth factor (NGF) receptor and its downstream target ERK1/2-type of mitogen-activated serine/threonine protein kinase are essential (12). Recently a critical role for a lipid kinase, phosphatidylinositol 3-kinase (PI 3-kinase), has been suggested in muscle and adipocyte differentiation (13, 14). Activation of PI 3-kinase is often associated with increased tyrosine phosphorylation induced by growth and differentiation factors (15). PI 3-kinase functions as the focal point in cellular signaling leading to cell growth, regulating cytoskeletal structure, and preventing apoptosis (16–18). Involvement of PI 3-kinase has recently been shown to

* This study was supported in part by the Dept. of Veterans Affairs Medical Research Service Merit Review Award, the Research Excellence Area Program (REAP) Award, National Institutes of Health Grant RO1 DK55815 (NIDDK) (to G. G. C.), and Dept. of Defense Breast Cancer Award DAMD17-99-1-9400 (to N. G. C.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

† Supported by a grant from the San Antonio Area Foundation and an institutional Howard Hughes grant.

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¶¶¶ Supported by National Institutes of Health Grant AR-42306.

1 The abbreviations used are: BMP, bone morphogenetic protein; BMPR, BMP receptor; PI, phosphatidylinositol; DN, dominant-negative; HA, hemagglutinin; TGF, transforming growth factor; GFP, green fluorescence protein; ERK, extracellular signal-regulated kinase; pNPP, p-nitrophenylphosphate; LUC, luciferase.
regulate some of the biological properties of TGF-β such as epithelial-mesenchymal transition and matrix protein expansion (19). However, the role of PI 3-kinase has not been investigated in osteogenesis, especially in response to BMPs. In this study, we show that BMP-2 stimulates tyrosine phosphorylation and PI 3-kinase activity in osteogenic cells. We demonstrate the requirement of PI-3 kinase and its downstream target, Akt serine/threonine kinase, for BMP-2-induced expression of an osteoblast differentiation marker alkaline phosphatase, and for BMP-2 transcription. Finally we demonstrate that a cross-talk exists between BMP-specific Smad and PI 3-kinase/Akt pathway to induce transcription of BMP-2, a necessary growth and differentiation factor for osteogenic cells.

EXPERIMENTAL PROCEDURES

Materials—Tissue culture reagents and LipofectAMINE were obtained from Invitrogen. Recombinant BMP-2 was obtained from the Genetics Institute. Nonidet P-40, phenylmethylsulfonyl fluoride, and Na3VO4 were purchased from Sigma. Aprotinin was obtained from Bayer. Antibody against the p85 subunit of PI 3-kinase, BMBRI, and Smads were purchased from Santa Cruz Biotechnology. Monoclonal anti-phosphotyrosine antibody (4G10) and Akt antibody were obtained from UBI Inc. Anti-hemagglutinin (HA) antibody was purchased from Babco. Histone H2B was purchased from Roche Molecular Biochemicals. Anti-phospho-Akt antibody was from Cell Signaling. Dual luciferase assay kit was purchased from Promega Inc. The pRcRap85 plasmid encoding for a dominant-negative (DN) subunit for PI-3 kinase was a gift from Dr. Joan Massague, Memorial Sloan-Kettering Cancer Center, and negative pCMV6-HA-Akt (K179M) expression vector was a kind gift from Dr. Thomas Franke, Harvard University. Smad1 plasmid was a gift from Dr. Joan Massague, Memorial Sloan-Kettering Cancer Center, NY. Adenovirus vectors containing constitutively active HA-tagged Alk3 Q233D (Ad-BMPPRIA-QD) and Alk6 Q203D (Ad-BMPRIIB-QD) were kindly provided by Anita Roberts, National Cancer Institute, MD.

Cell Culture and Adenovirus Infection—2T3 cells were isolated and cloned from a transgenic mouse with BMP-2 promoter-driven SV-40 T antigen (20). These cells are responsive to BMP-2, undergo bone matrix formation in vitro, and have been extensively characterized recently (20–23). 2T3 cells are routinely grown in α-minimum Eagle’s medium with 10% fetal bovine serum. The C2C12 multipotent cells were grown in Dulbecco’s modified Eagle’s medium in the presence of 10% fetal bovine serum. For osteoelastic differentiation, the cells were grown in the absence of serum, with or without recombinant BMP-2 as previously described (24). In experiments involving expression of constitutively active BMP receptor (BMPR), 2T3 cells in serum-free medium were infected with 100 m.o.i. Ad-BMPPRIA-QD or Ad-BMPRIIB-QD at room temperature for 1 h. Medium was changed with fresh serum-free medium. Similarly the 2T3 cells were infected with adenovirus vector expressing dominant-negative HA-tagged Akt (Ad-DN-Akt) essentially as described before (25). For all adenovirus infection, Ad-GFP containing green fluorescence protein was used as a control.

Immunoprecipitation, Immunoblotting, and PI 3-Kinase and Akt Kinase Assay—Cells were lysed in radioimmuno precipitation assay buffer (RIPA) (20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 5 mM EDTA, 1 mM Na3VO4, 1 mM phenylmethylsulfonyl fluoride, 0.05% aprotinin, and 1% Nonidet P-40) at 4 °C for 30 min. Cell extracts were centrifuged for 30 min at 4 °C. Protein concentration was determined in the supernatant using BioRad reagent. PI 3-kinase was assayed using PI as substrate in the presence of [γ-32P]ATP and the PI 3-phosphate was separated by thin-layer chromatography as described (26, 27). RIPA lysates were used to immunoprecipitate Akt, and immunocomplex kinase assay was performed with phosphotyrosine immunoprecipitates from lysates of BMP-2-treated 2T3 cells and analyzed by TLC. Arrow indicates position of PI 3-phosphate (PI-3-P).

RESULTS

Activation of PI 3-Kinase in Response to BMP-2—To investigate the role of PI 3-kinase in BMP-2-induced osteoblast differentiation, we used the 2T3 cells, which we established from the calvaria of a mouse expressing an SV-40 T-antigen transgene under transcriptional control of the BMP-2 promoter (20). This group and others (20–23) have shown that these cells turn on an osteoblast-specific gene program in response to BMP-2 and undergo differentiation to form mature osteoblasts.

Fig. 1. BMP-2 stimulates PI 3-kinase activity in 2T3 osteoblast precursor cells. A, serum-deprived 2T3 cells were incubated with BMP-2 for indicated periods of time. Cleared cell lysates were immunoprecipitated with anti-p85 antibody followed by PI 3-kinase assay as described under “Experimental Procedures.” The 3-phosphorylated PI (arrow) was separated by TLC. B, 50 μg of 2T3 cell lysates were analyzed by 10% SDS-polyacrylamide gel. Separated proteins were transferred to nitrocellulose membrane and immunoblotted with anti-phosphotyrosine antibody as described (25, 28–31). C, 500 μg of 2T3 cell lysate were immunoprecipitated with anti-p85 antibody followed by anti-phosphotyrosine immunoblotting. D, PI 3-kinase assay was performed with PI-3-kinase immunoprecipitates from lysates of BMP-2-treated 2T3 cells and analyzed by TLC. Arrow indicates position of PI 3-phosphate (PI-3-P).
cells were incubated with BMP-2 for different periods of time, and the lysates were immunoprecipitated with an antibody that recognizes the regulatory p85 subunit of PI 3-kinase. Immunocomplex PI 3-kinase assay showed that BMP-2 moderately increased PI 3-kinase activity in a time-dependent manner (Fig. 1A). The mechanism by which PI 3-kinase is activated by growth factors depends upon tyrosine phosphorylation of protein(s) where the SH2 domain of the regulatory p85 subunit of PI 3-kinase associates with specific phosphotyrosine (15). Therefore, we first tested the effect of BMP-2 on tyrosine phosphorylation of protein in 2T3 cells. Immunoblotting of BMP-2-treated 2T3 cell lysates showed increased tyrosine phosphorylation of multiple proteins to a variable extent (Fig. 1B). Although the regulatory p85 subunit of PI 3-kinase has been reported to be tyrosine-phosphorylated, BMP-2 did not induce tyrosine phosphorylation of any 85-kDa protein (Fig. 1B). However, anti-phosphotyrosine immunoblotting of p85 immunoprecipitates from BMP-2-treated 2T3 cell lysate showed increased association of the tyrosine-phosphorylated protein with the p85 regulatory subunit of PI 3-kinase (Fig. 1C). These data suggest that PI 3-kinase is present in a tyrosine-phosphorylated signaling complex in BMP-2-treated cells indicating a possibility where the lipid kinase can be activated by association with tyrosine-phosphorylated proteins in response to BMP-2. Anti-phosphotyrosine antibody was therefore used to immunoprecipitate and assay PI 3-kinase from lysates of BMP-2-treated 2T3 cells. BMP-2 significantly increased anti-phosphotyrosine-associated PI 3-kinase activity in a time-dependent manner (Fig. 1D) similar to that demonstrated in PI 3-kinase immunoprecipitates. Together these data indicate that BMP-2 regulates PI 3-kinase activity by stimulating association of p85 subunit of PI 3-kinase with a tyrosine-phosphorylated protein present in a signaling complex.

**Fig. 2. Inhibition of PI 3-kinase blocks BMP-2-induced alkaline phosphatase expression.** A, 2T3 cells were incubated with 12.5 μM Ly-294002 before treatment with BMP-2. Cell lysates were immunoprecipitated with anti-phosphotyrosine antibody followed by PI 3-kinase assay. Arrow indicates the position of PI 3-phosphate. B, 2T3 cells were treated with ME2SO vehicle or Ly-294002 prior to incubation with BMP-2 for 48 h. After fixation with formalin, the cells were stained with 5-bromo-4-chloro-3-indoyl phosphate and nitroblue tetrazolium for alkaline phosphatase as described (20). C, lysates from 2T3 cells incubated with Ly-294002 and BMP-2 were assayed for alkaline phosphatase activity using pNPP as substrate as described (32). Mean ± S.E. of triplicate measurements is shown. D, 2T3 cells were transfected with vector alone or pSRαp85 expressing the DN p85 subunit of PI 3-kinase. At 24-h post-transfection, the cells were treated with BMP-2 for 48 h and stained for alkaline phosphatase (20). Cells in panels a and c were incubated in the absence of BMP-2, and cells in panels b and d in the presence of BMP-2. E, lysates form dominant-negative PI 3-kinase transfected 2T3 cells, which were incubated with or without BMP-2, were assayed for alkaline phosphatase activity as described (32).
BMP-2-induced PI 3-kinase activity (Fig. 2A, compare lane 4 with lane 2). To test the effect of PI 3-kinase on BMP-2-mediated alkaline phosphatase expression, we treated 2T3 cells with BMP-2 in the presence and absence of Ly-294002 and stained for alkaline phosphatase activity. As expected, BMP-2 increased expression of alkaline phosphatase (Fig. 2B, compare panel b with panel a). Inhibition of PI 3-kinase activity by Ly-294002 significantly blocked BMP-2-induced alkaline phosphatase (Fig. 2B, compare panel d with panel b). Similarly, expression of dominant-negative p85 subunit of PI 3-kinase significantly blocked alkaline phosphatase activity in the lysates of 2T3 cells (Fig. 2E). These data indicate that PI 3-kinase-dependent signal transduction regulates BMP-2-induced expression of alkaline phosphatase.

BMP-2 Stimulates Akt Serine/Threonine Kinase—Although PI 3-kinase-independent activation of Akt has been documented, one of the principal downstream targets of PI 3-kinase is the serine/threonine kinase Akt (35, 36). Phosphorylation of Akt by 3-phosphoinositide-dependent kinases 1 and 2 represents the activated state of this enzyme (37). To investigate whether BMP-2-induced osteoblast differentiation is mediated by Akt, we investigated the effect of BMP-2 on activation of Akt in 2T3 cells. Activation was determined by immunoblot analysis of lysates from BMP-2-treated 2T3 cells using an anti-phospho-Akt-specific antibody that recognizes the activated form. Akt was activated in response to BMP-2 in a time-dependent manner (Fig. 3A). The kinetics of BMP-2-induced Akt activation were similar to those of BMP-2-induced PI 3-kinase activity in these cells (Fig. 1), suggesting that activation of Akt may be PI 3-kinase-dependent. To test this directly, we treated 2T3 cells with Ly-294002 followed by BMP-2. Similar to Akt activation determined by anti-phospho-Akt antibody (Fig. 3A), BMP-2 stimulated Akt kinase activity as determined by Akt immunocomplex kinase assay using histone H2B as substrate (Fig. 3B). Inhibition of PI 3-kinase by Ly-294002 abolished BMP-2-induced Akt activity (Fig. 3B, compare lane 4 with lane 2). These data indicate that BMP-2 stimulates Akt kinase via activation of PI 3-kinase.

Akt Regulates BMP-2-induced Alkaline Phosphatase Expression—Because PI 3-kinase is required for BMP-2-induced alkaline phosphatase activity (Fig. 2), we investigated whether the downstream target of PI 3-kinase, Akt serine/threonine kinase, regulates this process. We transfected a kinase-deficient Akt, which this group and others (25, 36) have shown previously to act in a dominant-negative fashion, into 2T3 cells. Transfected cells were then incubated with BMP-2, and expression of alkaline phosphatase was tested by activity staining. Expression of dominant-negative Akt significantly blocked catalytic p110 subunit of PI 3-kinase (34). The cells were then treated with BMP-2, and expression of alkaline phosphatase was tested by activity staining. Expression of dominant-negative PI 3-kinase significantly blocked BMP-2-induced alkaline phosphatase expression (Fig. 2D, compare panel d with panel b). Similarly, expression of dominant-negative p85 subunit of PI 3-kinase significantly blocked alkaline phosphatase activity in the lysates of 2T3 cells (Fig. 2E). These data indicate that PI 3-kinase-dependent signal transduction regulates BMP-2-induced expression of alkaline phosphatase.
BMP-2-induced alkaline phosphatase staining (Fig. 4A, compare panel d with panel b). To confirm this observation, the alkaline phosphatase activity in the lysates was measured. Expression of dominant-negative Akt inhibited alkaline phosphatase activity (Fig. 4B). Together these data indicate that BMP-2-stimulated PI 3-kinase/Akt signaling cascade regulates alkaline phosphatase, an early marker of osteoblast differentiation.

**BMPRIA and BMPRIB Regulate PI 3-Kinase and Akt Activities**—In the activated oligomeric receptor complex, BMPRI acts as the signal transmitter (6, 38). Differential action of BMPRIA and BMPRIB has been reported during vertebrate embryonic development and chick limb bud development (39, 40). To examine whether PI 3-kinase and Akt are stimulated by both these receptors, we used adenovirus vectors containing constitutively active BMPRIA (Ad-BMPRIA-QD) and BMPRIB (Ad-BMPRIB-QD). Infection of 2T3 cells with these vectors showed significant levels of expression of both these receptors (Fig. 5A). As expected, BMPRIA migrated slightly slower than did BMPRIB (Fig. 5A) (41, 42). Constitutively active receptor expression was detectable as early as 6 h of vector infection (Fig. 5, B and C). Therefore, we used anti-phosphotyrosine immunoprecipitates from these adenovirus vector-infected 2T3 cells for PI 3-kinase activity. Expression of constitutively active BMPRIA and BMPRIB significantly activated PI 3-kinase activity, respectively (Fig. 5B, top panel, compare lanes 1 and 3 with lane 2, respectively). Because Akt is downstream of BMP-2-stimulated PI 3-kinase (Fig. 3), immunocomplex kinase assay of Akt immunoprecipitates from lysate of Ad-BMPRIA-QD- and Ad-BMPRIB-QD-infected 2T3 cells was performed. Both BMPRIA and BMPRIB significantly increased Akt kinase activity (Fig. 5C, top panel, compare lanes 2 and 3 with lane 1). These data indicate that in osteoblasts both type I receptors are capable of contributing in activation of PI 3-kinase/Akt signaling.

**PI 3-Kinase and Akt Regulate BMP-2-induced BMP-2 Transcription**—We previously reported that BMP-2 stimulates BMP-2 gene expression by increased transcription during osteoblast differentiation (3, 20, 33). This autoregulation of BMP-2 expression maintains its sustained effect on terminally differentiated osteoblasts. Because mechanisms of signaling of BMP-2 transcription are not known, we investigated the role of PI 3-kinase in BMP-2 gene transcription. A reporter construct in which the BMP-2 promoter drives firefly luciferase cDNA (BMP-2-LUC) was transfected either with dominant-negative p85 subunit of PI 3-kinase or with kinase-dead Akt into 2T3 cells. Incubation of transiently transfected cells with BMP-2 increased the reporter gene expression indicating that BMP-2 autoregulates its own transcription (Fig. 6, A and B). However, transfection of dominant-negative PI 3-kinase or dominant-negative Akt significantly blocked BMP-2-induced BMP-2 transcription (Fig. 6, A and B, respectively). These data indicate that PI 3-kinase/Akt signaling regulates BMP-2 gene transcription induced by BMP-2.

All the known biological activities of BMP-2 are mediated by BMP-specific Smad proteins (4, 7). BMP-activated Smad, such as Smad5, stimulates transcription of target genes required for osteoblast differentiation. Because BMP-2 stimulates transcription of the BMP-2 gene (20, 33), we investigated the effect of Smad5 on BMP-2 promoter activity in 2T3 cells using the BMP-2-LUC reporter construct. Transfection of Smad5 alone increased BMP-2 gene transcription in these cells in a ligand-independent manner (Fig. 6, C and D). Transfection of dominant-negative PI 3-kinase blocked Smad5-induced transcription.
tion of BMP-2 promoter-driven reporter gene (Fig. 6C). Similarly, transfection of dominant-negative Akt also inhibited Smad5-dependent transcription of BMP-2 promoter (Fig. 6D). These data provide evidence that PI 3-kinase/Akt signal transduction pathway integrates into the nucleus to influence Smad-mediated transcription.

Next we investigated the mechanism by which Akt may regulate BMP-2-induced Smad-dependent transcription. Analysis of the primary amino acid sequence of Smad5 and Smad1 revealed absence of any consensus Akt phosphorylation site (RXXRX(S/T)). The mechanism by which BMP regulates Smad function is by inducing translocation of these cytosolic proteins into the nucleus leading to transcription of target genes (4, 5, 7). To examine whether Akt regulates Smad translocation, we infected 2T3 cells with Ad-DN-Akt, an adenovirus vector containing HA-tagged dominant-negative Akt, followed by incubation with BMP-2. Translocation of BMP-specific Smad was studied by immunofluorescence using an antibody that recognizes both Smad5 and Smad1. As expected BMP-2 stimulated translocation of cytosolic and perinuclear Smad into the nucleus with punctate appearance on a diffused nuclear staining (Fig. 7, compare panel B with panel A) (43). Expression of dominant-negative Akt significantly blocked BMP-2-induced nuclear localization of Smad (Fig. 7, compare panel D with panel B). These data indicate that Akt, in the absence of its consensus phosphorylation site, regulates translocation of BMP-specific Smads into the nucleus, and hence indirectly modulates Smad-dependent transcription.

**DISCUSSION**

These studies represent the first demonstration of activation of the PI 3-kinase/Akt pathway in response to the osteogenic factor BMP-2. We demonstrate that BMP-2-induced expression of alkaline phosphatase, an enzyme expressed during osteoblast differentiation of progenitor cells into mature osteoblast, requires activation of PI 3-kinase and its downstream target Akt serine/threonine kinase. Our data also provide the first evidence that PI 3-kinase and Akt modulate autoregulation of BMP-2 gene transcription and that a cross-talk exists between the BMP-specific Smad and PI 3-kinase/Akt signaling pathway for regulation of BMP-2 transcription.

Activation of PI 3-kinase regulates cellular processes including proliferation, migration, secretion, endocytosis, and protein transport (17). Recently a role of PI 3-kinase has been shown in TGF-β-induced epithelial and endothelial cell survival and epithelial to mesenchymal transition (19, 44, 45) indicating that activation of serine/threonine kinase receptor utilizes this central lipid kinase as one of the signaling mechanisms similar to receptor tyrosine kinases. Recently PI 3-kinase has been implicated in myogenic differentiation (13, 46, 47). Also insulin-induced adipocyte differentiation utilizes activation of PI 3-kinase pathway (48, 49). We have shown here that the osteogenic differentiation factor BMP-2 stimulates PI 3-kinase activity (Fig. 1). Increased PI 3-kinase activity is often associated with receptor and non-receptor tyrosine kinase-mediated signal transduction in which activation of this lipid kinase is a result...
of its association with specific tyrosine-phosphorylated proteins (15). In this report we demonstrate that activation of the BMP receptor results in increased tyrosine phosphorylation of proteins leading to association of the regulatory subunit of PI 3-kinase with tyrosine-phosphorylated proteins (Fig. 1, B and C). Also, we document BMP-2 induced PI 3-kinase activity in the tyrosine-phosphorylated protein fraction (Fig. 1D). Additionally we demonstrate that both BMPRIA and BMPRIB are involved in activation of PI 3-kinase in the tyrosine-phosphorylated protein fraction (Fig. 5). However, we were unable to detect any association of PI 3-kinase with the BMPRI in the BMPRI immunoprecipitates (data not shown). These data indicate that not only receptor tyrosine kinases, but serine/threonine kinase receptor utilizes the similar tyrosine phosphorylation mechanism to stimulate PI 3-kinase activity. It is evident from our data that the PI 3-kinase activity in p85 immunoprecipitates is somewhat less (Fig. 1A) than that in anti-phosphotyrosine immunoprecipitates (Fig. 1D). One reason for this may be that p85 antibody recognizes the total pool of PI 3-kinase present in the cell whereas anti-phosphotyrosine antibody recognizes only the activated form of PI 3-kinase, which is associated with tyrosin-phosphorylated proteins in response to BMP-2.

Differentiation of cells is always associated with induction of specific protein markers resulting from cell- and differentiation-specific transcriptional programs. Thus a hallmark of osteoblast differentiation is expression of alkaline phosphatase, which is induced early in the differentiation process (3, 20, 32). We showed previously that BMP-2 stimulated alkaline phosphatase mRNA and protein expression in 2T3 cells during the initiation of differentiation (3, 20). Using a PI 3-kinase inhibitor, we have now demonstrated that activation of this lipid kinase is essential for BMP-2-induced alkaline phosphatase activity in these cells (Fig. 2, A–C). Furthermore the absence of alkaline phosphatase activity in 2T3 cells expressing a dominant-negative PI 3-kinase conclusively establishes the requirement of PI 3-kinase for this osteoblast differentiation-specific early enzyme activity (Fig. 2, D and E). In addition, in long term culture, incubation of 2T3 cells with PI 3-kinase inhibitor Ly-294002 in the presence of BMP-2 completely blocked mineralized bone nodule formation indicating further the importance of this lipid kinase in osteoblast differentiation (data not shown).

Many biological functions of PI 3-kinase are regulated by its direct downstream target, Akt serine/threonine kinase (16, 25, 35). The products of PI 3-kinase, the D3-phosphoinositides, bind to the N-terminal plecrtin homology (PH) domain of Akt to recruit it into the plasma membrane where PDK1 and PDK2 phosphorylate it, resulting in its full activation (37, 50). Although a diverse group of proteins that function in many different cellular processes has been identified as substrates of Akt, the role of Akt in inhibition of apoptosis has been most extensively studied (35). This action of Akt is mediated by phosphorylation of pro-apoptotic protein BAD and caspase 9 (51, 52). Terminal cellular differentiation is a post-mitotic phenomenon where cells acquire an apoptosis-resistant phenotype (53). Recently Akt has been implicated in myogenic differentiation (54, 55). Also Akt regulates IGF-induced myotube formation, as a downstream target of PI 3-kinase. This action of Akt is mediated by phosphorylation of Raf-1, which negatively regulates its kinase activity and results in maintenance of highly differentiated myotubes (56). Here we show that the osteogenic factor BMP-2 stimulates Akt activity in a PI 3-kinase-dependent manner in 2T3 osteoblast precursor cells in response to BMP-2 (Fig. 3). Expression of dominant-negative Akt resulted in significant inhibition of BMP-2-induced alkaline phosphatase activity in these cells, indicating that Akt regulates the expression of this early osteoblast-specific marker protein (Fig. 4). Along with 2T3 cells, which we established and used extensively as a model for osteoblast growth and differentiation (3, 20–23), we used another multipotent cell line, C2C12, which has the potential to undergo osteoblast differentiation in the presence of BMP-2 (24, 57). Similar to 2T3 cells, BMP-2 stimulated Akt kinase activity in these cells, and expression of
dominant-negative Akt significantly blocked BMP-2-induced alkaline phosphatase activity (data not shown). These results further confirm our observation that PI 3-kinase/Akt signaling cascade regulates osteoblast differentiation in cells, which have the potential to undergo differentiation to osteoblasts in response to BMP-2.

BMP-regulated Smad1 or Smad5 after dimerization with BMPRIA and BMPRIB.

...critisitively reading the manuscript. We also thank Drs. Thomas Franke, M. T. F. Kaplan, D. R. & Cantley, L. C. (1997) Cell, 88, 435–437.
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