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Inhibition of Phosphatidylinositol 3-Kinase and p70S6 Kinase Blocks Osteogenic Protein-1 Induction of Alkaline Phosphatase Activity in Fetal Rat Calvaria Cells

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Abstract Published studies reveal that Osteogenic Protein-1 (OP-1) and insulin-like growth factor-I (IGF-I) synergistically stimulate alkaline phosphatase (AP) activity and bone nodule formation in fetal rat calvaria (FRC) cells. In the present study, we examined whether there are interactions between the signal transduction pathways activated by these two growth factors. OP-1 did not significantly affect the levels of IRS-1, IRS-2, the p85α subunit of phosphatidylinositol 3-kinase (PI 3-kinase) or the extracellular signal-regulated kinase (ERK)-2, but stimulated ERK-1 protein by twofold. OP-1 also induced phosphorylation of ERK-1 and -2, but not of Akt/protein kinase B (PKB), a protein kinase that is downstream of PI 3-kinase. By comparison, IGF-I increased the levels of the phosphorylated forms of ERK-1 and -2, and Akt/PKB. Inhibition of ERK activation by PD98059 did not significantly alter the stimulation of AP activity by OP-1 or OP-1 in combination with IGF-I. In contrast, inhibition of PI 3-kinase activity by LY294002 blocked the induction of AP activity by OP-1 and OP-1 plus IGF-I. Treatment of cells with rapamycin, an inhibitor of the mammalian target of mTOR, resulted in a 47% and a 53% decrease in the AP activity induced by OP-1 alone and by OP-1 plus IGF-I, respectively. These studies suggest that PI 3-kinase and mTOR contribute to the induction of AP activity by OP-1 and the synergistic effect of OP-1 and IGF-I on AP activity in FRC cells. J. Cell. Biochem. 88: 1247–1255, 2003.

Key words: IGF-I; BMP; osteogenic protein-1; signal transduction; osteoblastic cell

Osteogenic Protein-1 (OP-1, BMP-7) is a member of the bone morphogenetic protein (BMP) subfamily of the transforming growth factor-β (TGF-β) superfamily [Wozney et al., 1988; Ozkaynak et al., 1990; Sampath et al., 1990]. OP-1 has been shown to induce bone formation in vivo [Sampath et al., 1990, 1992] and stimulates synthesis of biochemical markers that are characteristic of osteoblast differentiation in vitro. These markers include alkaline phosphatase (AP), osteopontin, bone sialoprotein, and osteocalcin [Sampath et al., 1992; Asahina et al., 1993; Knutsen et al., 1993; Kitten et al., 1995; Sodek et al., 1995; Yeh et al., 2000]. In primary cultures of fetal rat calvaria (FRC) cells, OP-1 stimulates differentiation of confluent FRC cells into an osteoblastic phenotype [Asahina et al., 1993; Yeh et al., 1997].

Insulin-like growth factor-I (IGF-I) enhanced the OP-1-induced AP activity and bone nodule formation in FRC cells [Yeh et al., 1997]. This synergy was observed only when IGF-I was added to FRC cells simultaneously or within 6 h of OP-1 treatment, suggesting that IGF-I acts on OP-1 sensitized FRC cells. Further studies revealed that inhibition of IGF-I synthesis using antisense RNA resulted in a partial reduction in the OP-1-induced AP activity [Yeh et al., 1996]. Moreover, OP-1 has been shown to influence the protein and mRNA expression of the components of the IGF system in different osteoblastic cell lines and primary cultures of fetal rat calvaria cells. The components affected include IGF-I and -II [Knutsen et al., 1995; Yeh et al., 1997, 1998], and IGF binding proteins (IGFBPs) -3, -4, -5, and -6 [Yeh et al., 1996, 1997, 1998; Yeh and Lee, 2000]. OP-1 did not

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significantly increase IGF-I receptor mRNA expression [Yeh et al., 1996]. However, very little is known about the effect of OP-1 on the expression of the components of the IGF-I signal transduction pathways.

Since IGF-I did not induce AP activity independently of OP-1 in FRC cells [Yeh et al., 1997], we postulated that OP-1 potentiates the response of FRC cells to IGF-I by regulating the expression or activation of specific components of the IGF-I signaling machinery. These include the extracellular signal-regulated kinase (ERK)-1/-2 pathway [Coolican et al., 1997] and the PI 3-kinase pathway [Myers et al., 1993; Giorgino and Smith, 1995]. The present report describes the results of our study on effects of OP-1 and OP-1 plus IGF-I on these signaling pathways.

MATERIALS AND METHODS

Reagents and Antibodies

Hanks balanced salt solution (HBSS), fetal bovine serum (FBS), serum-free αMEM medium, penicillin/streptomycin stock, trypsin-EDTA (0.05% trypsin-0.53 mM EDTA), and collagenase were obtained from Life Technologies (Grand Island, NY). Recombinant human OP-1 was provided by Stryker Biotech (Hopkinton, MA), dissolved in 47.5% ethanol/0.01% trifluoroacetic acid, and stored at −20°C. Human IGF-I was obtained from Life Technologies, dissolved in 0.1 M acetic acid, and stored in aliquots (100 ng/μl) at −20°C. LY294002, PD98059, and rapamycin were obtained from Calbiochem (La Jolla, CA), dissolved in DMSO (30 mM, 30 mM, and 1 mM, respectively), and stored in aliquots at −20°C. Mouse monoclonal antibodies specific against IRS-1, and p85α subunit of PI 3-kinase were obtained from Transduction Laboratories (Lexington, KY). Rabbit polyclonal antibodies specific for IRS-2 were obtained from Upstate Biotechnology (Lake Placid, NY). Rabbit polyclonal antibodies that were specific for the phosphorylated and the unphosphorylated forms of Akt/PKB and ERK-1 and -2 were obtained from Cell Signaling Technology (Beverly, MA).

Preparation and Culture of Fetal Rat Calvaria Cells

Primary osteoblast cell cultures were prepared from calvaria of 19-day-old fetal rats using previously described methods [McCarthy et al., 1988; Aronow et al., 1990; Yeh et al., 1996]. Briefly, the cleaned calvarium was digested with a mixture of trypsin and collagenase for five 20-min intervals. The digestion mixture from each 20-min interval was collected separately. FRC cells were harvested from digestions 3–5. Cells were plated in T-75 flasks at a density of $2.5 \times 10^4/cm^2$ in complete αMEM medium containing 10% FBS, vitamin C (100 μg/ml), 100 U/ml penicillin, and 100 μg/ml streptomycin sulfate. Cells were incubated at 37°C with 5% CO_2. For experimentation, confluent FRC cells (passage 3) were incubated in the serum-free αMEM medium (with 0.1% BSA) in the absence or presence of OP-1 (200 ng/ml) and without or with IGF-I (25 ng/ml) as described in the figure legends. For inhibitor studies, FRC cells were pre-treated with either PD98059, LY294002, or rapamycin for 30 min, and then treated without or with OP-1 (200 ng/ml), or IGF-1 (25 ng/ml), or the combination of OP-1 (200 ng/ml) and IGF-I (25 ng/ml) for 48 h, in the presence or absence of PD98059 (15 μM), LY294002 (10 μM), or rapamycin (109 nM).

AP Activity Assay

FRC cells were grown and treated in 48-well plates as described above. The medium was replenished with fresh medium after 8, 24, and 32 h of treatment to ensure a continuous supply of OP-1, IGF-I, nutrients, and inhibitors. After 48 h of treatment, cells were rinsed with PBS and lysed by sonication in 0.05% Triton X-100 in PBS (100 μl/ well) for 60 sec at room temperature. Total cellular AP activity was measured with *p*-nitrophenyl phosphate as a substrate at 37°C using a commercial assay kit (Sigma Chemical Co.). Reactions were terminated by the addition of 0.5 N NaOH. Absorbance of the reaction mixture was measured at 405 nm using a MRX microplate reader (Dynatec Laboratories, Inc., Chantilly, VA). Protein was measured according to the method of Bradford [1976], using BSA as a standard. AP activity was expressed as nanomoles of *p*-nitrophenol liberated per microgram total cellular protein.

Western Blot Analyses

FRC cells were grown and treated in 6-well plates as described above. Cells were washed twice in PBS and lysed in 50 mM Tris pH 7.4, 1 mM EDTA, 150 mM NaCl, 0.1% (v/v) glycerol, 0.01% (v/v) NP-40, 0.005% (v/v) Triton X-100, 0.1 M phenylmethylsulfonyl fluoride (PMSF),

0.4 μg/ml pepstatin A, 0.2 μg/ml leupeptin, 0.2 μg/ml antipain HC, 0.2 μg/ml chymostatin, 1 mM sodium orthovanadate, 2 mM sodium pyrophosphate, and 25 mM sodium fluoride. Lysates were clarified by centrifugation at 15,000*g* for 5 min at 4°C, and protein concentrations were determined according to the method of Bradford. Proteins were separated by SDS-PAGE in a 7.5% polyacrylamide gel. Pre-stained molecular weight markers (Bio-Rad) were used as standards. Proteins were transferred to PVDF membranes (Immobilon-P, 0.45 μm, Millipore Corporation, Bedford, MA) in 25 mM Tris-HCl, 192 mM glycine and 20% methanol with a semi-dry Trans-Blot transfer system (Bio-Rad). Membranes were blocked for 90 min at room temperature in 20 mM Tris, pH 7.6, 137 mM sodium chloride, 0.1% Tween-20 (TBST), and 1% nonfat dried milk. Membranes were incubated overnight in TBST containing 5% BSA at 4°C with the primary antibody and then with anti-mouse or anti-rabbit (Promega Corporation, Madison, WI) immunoglobulin G horseradish peroxidase-conjugated antibodies for 90 min at room temperature. Following three washes in TBST, immunoreactive bands were detected using the SuperSignal chemiluminescent detection system (Pierce, Rockford, IL), according to the manufacturer's instructions. Results represent the average from two independent experiments. The band intensities were quantified using the Image-Quant Software (Molecular Dynamics).

Statistical Analysis

Data are expressed as the mean ± SEM. Statistical evaluation of the data was performed using one-way ANOVA followed by post hoc comparison in the SIMSTAT3 package (Normand Peladeau, Provalis Research, Montreal, Canada). Differences among means were considered significant when $P < 0.05$.

RESULTS

OP-1 Exerted Differential Effects on the Expression Levels of the IGF-I Signal Transduction Pathway Molecules

To examine whether OP-1 regulates the expression level of IGF-I signaling molecules, FRC cells were treated with or without OP-1 for varying periods of time, followed by Western blot analyses. IRS-1, a specific substrate of the IGF-I and insulin receptors that acts upstream of PI 3-kinase [Myers et al., 1993] was not detected in untreated FRC cells, and its expression was not induced by OP-1 (data not shown). IRS-2, an alternative of IRS-1 in IGF-I signaling [Valverde et al., 1998] was detected in untreated FRC cells, and its level was not affected by OP-1 treatment (data not shown). The p85α subunit of PI 3-kinase was detectable in untreated FRC cells, but its level was not changed in OP-1-treated samples (data not shown). Both ERK-1 and -2 were detected in untreated FRC cells. OP-1 treatment stimulated ERK-1 protein expression by twofold, compared to IRS-2 at 3 h (Fig. 1A), and the level remained elevated above the control at 6 h. The ERK-2 levels were higher than the ERK-1 in control cultures. The ERK-2 levels remained relatively unchanged in the control and the OP-1-treated samples (Fig. 1B), except the 6 h treated sample, which showed a 32% increase ($P < 0.05$).

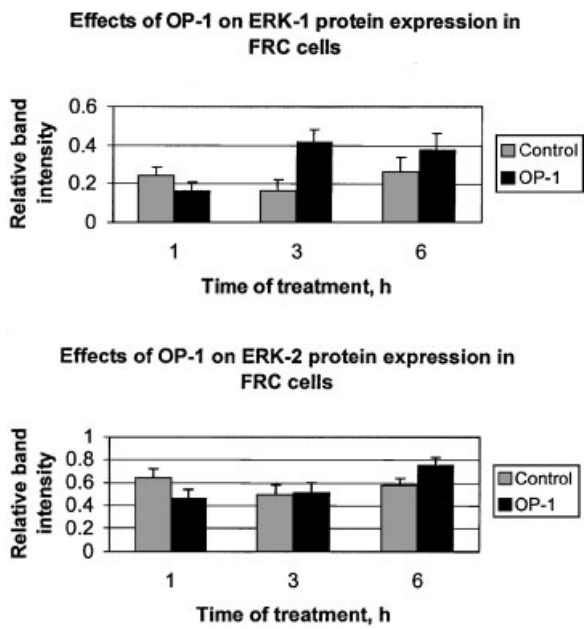

Fig. 1. Effects of Osteogenic Protein-1 (OP-1) on the levels of expression of molecules that mediate insulin-like growth factor-I (IGF-I) initiated signal transduction in fetal rat calvaria (FRC) cells. FRC cells were treated with or without OP-1 for 1–6 h. Total cellular protein was prepared (as described in Materials and Methods), and subjected to Western blot analyses using antibodies against extracellular signal-regulated kinases (ERKs). Band intensity was measured using the ImageQuant Software. Values are the mean ± SEM of two to three independent experiments; each performed using protein prepared from a different preparation of FRC cells.

IGF-I and OP-1 Induced Phosphorylation of ERK-1 and -2 in FRC Cells

The above data suggest that ERK protein expression was affected by OP-1 at the early time points. To examine further whether OP-1 and IGF-I activate the same pathways in FRC cells at the early time period, Western blot analyses were performed using antibodies that were specific for the phosphorylated forms of ERK-1 and -2. The quantitative data are shown in Figure 2 in which the level of phospho-ERK is normalized to the total ERK. Because no significant differences were observed between the extent of phosphorylation of ERK-1 and -2, total phospho-ERK (pERK-1 plus pERK-2) is shown. Both IGF-I and OP-1 stimulated phosphorylation of ERK-1 and -2 by about threefold within 5 min. The level remained elevated at 15 min, but declined nearly to the control by 30 min (Fig. 2). After 2 h of IGF-I treatment, neither phosphorylated forms could be detected in untreated samples, but significant levels of both forms were detected in the OP-1-treated samples.

PD98059 Did Not Affect the Synergistic Induction of AP Activity by OP-1 and IGF-I

The above results show that the levels of ERK-1 were regulated by OP-1, and that the ERK pathway is activated by both OP-1 and IGF-I. We examined whether the ERKs are involved in the previously observed synergistic induction of AP activity by OP-1 and IGF-I [Yeh et al., 1997], using PD98059, a pharmacological compound that inhibits MEK, thereby inhibiting the activation of the ERKs [Alessi et al., 1995; Dudley et al., 1995]. Accordingly, FRC cells were pretreated with PD98059 followed by OP-1, IGF-I, or the combination of OP-1 and IGF-I for 48 h. An examination of these cells under a phase contrast microscope at the end of the treatment period failed to reveal detectable changes in cell growth or necrosis. Consistent with our published results, OP-1 induced AP activity by about threefold, compared to the control (Fig. 3A, lane 2 vs. lane 1). The induced-AP activity was enhanced by IGF-I resulting in a fourfold increase, compared to the control (Fig. 3A, lane 4 vs. lane 2). IGF-I alone did not induce AP activity (lane 3 vs. lane 1). PD98059 did not significantly affect the basal AP activity, the OP-1-induced AP activity (lane 6 vs. lane 2), or the activity induced by OP-1 in the presence of IGF-I (lane 8 vs. lane 4).

To confirm that this concentration of PD98059 was sufficient to inhibit ERK phosphorylation, FRC cells were pretreated with or without PD98059 and then treated with IGF-I for 15 min in the presence or absence of PD98059. Western blot analyses showed that PD98059 inhibited both the basal and the IGF-I-induced ERK-1 and -2 phosphorylation (Fig. 3B). Levels of total ERK-1 and -2 were similar in all samples.

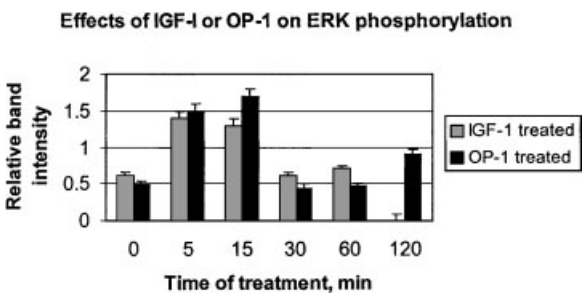

Fig. 2. Osteogenic Protein-1 (OP-1) and insulin-like growth factor-I (IGF-I) induced phosphorylation of extracellular signal-regulated kinase (ERK)-1 and -2 in fetal rat calvaria (FRC) cells. FRC cells were treated with or without 25 ng/ml IGF-I or 200 ng/ml OP-1 for 5 to 120 min. Total cellular protein was prepared and subjected to Western blot analyses using antibodies directed against phospho-ERK and total-ERK. Band intensity was measured using the ImageQuant Software. Values are the mean ± SEM of two independent experiments; each performed using protein prepared from a different preparation of FRC cells.

IGF-I but not OP-1, Induced Phosphorylation of Akt/PKB and OP-1 Did Not Change the IGF-I Induced Akt/PKB Phosphorylation in FRC Cells

To determine whether OP-1 affects the other IGF-I signaling pathway, viz., the PI 3-kinase pathway, Western blot analyses were performed using antibodies specific for the phosphorylated forms of Akt/PKB, a protein kinase that functions downstream of PI 3-kinase [Burgering and Coffer, 1995]. IGF-I stimulated Akt phosphorylation within 5 min and persisted up to 120 min (Fig. 4A). In contrast, OP-1 did not induce Akt phosphorylation in FRC cells (Fig. 4C), or alter the IGF-I stimulated Akt phosphorylation (data not shown). The levels of total Akt were relatively the same in all samples (Fig. 4B,D).

LY294002 Inhibited the OP-1-Induced AP Activity and Reduced the Synergistic Effect of OP-1 and IGF-I

To evaluate whether PI 3-kinase contributes to the synergistic induction of AP activity by

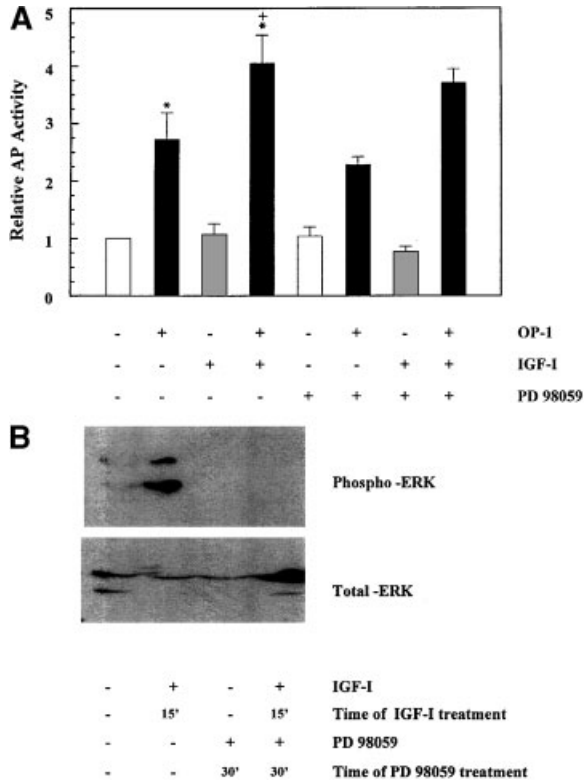

Fig. 3. Effects of PD98059 on the synergistic induction of alkaline phosphatase (AP) activity by Osteogenic Protein-1 (OP-1) and insulin-like growth factor-I (IGF-I) in fetal rat calvaria (FRC) cells. **A**: FRC cells were pretreated with PD98059 (15 μM) for 30 min and then treated with vehicle or OP-1 (200 ng/ml), or IGF-I (25 ng/ml) or the combination of OP-1 (200 ng/ml) and IGF-I (25 ng/ml) for 48 h in the presence or absence of PD98059 (15 μM). The medium was changed every 8 to 16 h. Total AP activity was measured after 48 h. Values represent the mean ± SE of five independent determinations from three different FRC preparations. The means were normalized to the control, which was defined as 1. $^{*}P < 0.01$ compared to the level of AP activity in control cells. $^{+}P < 0.05$ compared to the AP activity in OP-1-treated sample. **B**: FRC cells were pretreated with PD98059 (15 μM) for 30 min and then treated with vehicle or IGF-I (25 ng/ml) for 0.5 min to 48 h in the presence or absence of PD98059 (15 μM). Lysate that was prepared and subjected to Western blot analysis using an antibody directed against phospho-extracellular signal-regulated kinase (ERK) or total ERK. These results are representatives of two independent experiments, each using protein extracts obtained from independent preparations of FRC cells.

OP-1 and IGF-I, FRC cells were pretreated with or without LY294002, an inhibitor of PI 3-kinase activity [Vlahos et al., 1994] followed by OP-1, IGF-1, or the combination of OP-1 and IGF-I for 48 h, in the presence or absence of LY294002. At the end of the treatment period, these cells did not reveal detectable changes in cell growth or necrosis as examined under a phase contrast microscope. LY294002 did not change the basal levels of AP activity (Fig. 5, lane 5 vs. lane 1), or the AP activity in the IGF-I treated samples (lane 7 vs. lane 3). However, LY294002 inhibited the AP activity induced by OP-1 alone (lane 6 vs. lane 2) and OP-1 plus IGF-I (lane 8 vs. lane 4) by 88% and 83%, respectively.

Rapamycin Reduced the Synergistic Induction of AP Activity by OP-1 and IGF-I

The above results showed that PI 3-kinase contributed to the synergistic induction of AP activity by OP-1 and IGF-I. We then examined whether p70S6 kinase, a downstream mediator of PI 3-kinase activity [Cheatham et al., 1994] might be responsible for the synergistic effect. The effect of rapamycin, a pharmacological compound that inhibits mTOR, thereby inhibiting the activation of p70S6 kinase [Price et al., 1992] on the induced-AP activity was examined. FRC cells were pretreated with or without rapamycin, and then treated without or with OP-1, IGF-I, or the combination of OP-1 and IGF-I for 48 h, in the presence or absence of rapamycin. An examination of these cells under a phase contrast microscope at the end of the treatment period did not reveal detectable changes in cell growth or necrosis. Rapamycin did not change the basal level of AP activity (Fig. 6, lane 5 vs. lane 1) and the AP activity in the IGF-I treated samples (Fig. 6, lane 7 vs. lane 3). However, rapamycin reduced the OP-1-induced AP activity by 47% (lane 6 vs. lane 2, $P < 0.06$), and the synergistic induction of AP activity by OP-1 and IGF-I by 53% (lane 8 vs. lane 4, $P < 0.003$).

DISCUSSION

Previous studies showed that OP-1 and IGF-I are important for osteoblast differentiation and bone formation [Hock et al., 1988; Sampath et al., 1990, 1992; Yeh et al., 1997]. It was further shown that IGF-I enhanced the OP-1-induced AP activity [Yeh et al., 1997]. This synergy was suggested to be partially due to regulation of gene expression of several of the IGF system components by OP-1 [Yeh et al., 1996, 1997].

The present study examined the effects of OP-1 on the expression of the IGF-I signaling molecules and showed that OP-1 did not significantly affect the level of expression of certain molecules that are known to mediate IGF-I

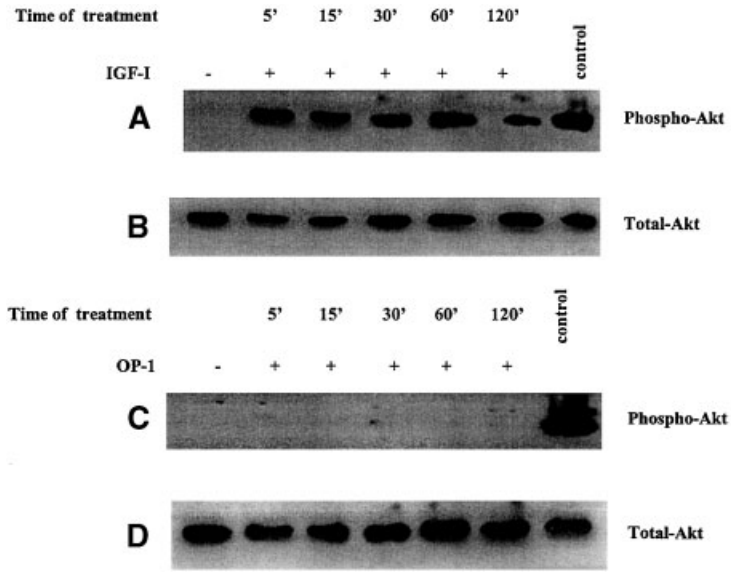

Fig. 4. Insulin-like growth factor-I (IGF-I) but not Osteogenic Protein-1 (OP-1) induced phosphorylation of Akt/PKB in fetal rat calvaria (FRC) cells. FRC cells were treated with (+) or without (−) 25 ng/ml IGF-I or 200 ng/ml OP-1 for 5 to 120 min. Western blot analysis was carried out as described in Figure 2, except the following antibodies were used: phospho-Akt (**A**,**C**), and total-Akt (**B**,**D**). The control sample shown contained proteins from C6 cells that express constitutively phosphorylated Akt [Wang et al., 2001]. The data are representative of results obtained from two independent experiments; each performed using protein prepared from a different preparation of FRC cells.

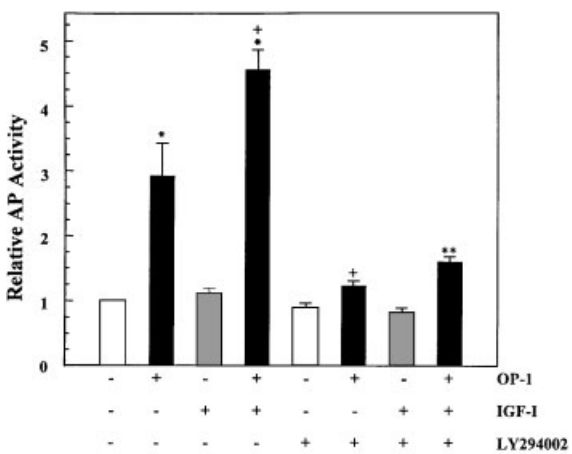

Fig. 5. Effects of LY294002 on the synergistic induction of alkaline phosphatase (AP) activity by Osteogenic Protein-1 (OP-1) and insulin-like growth factor-I (IGF-I) in fetal rat calvaria (FRC) cells. FRC cells were pretreated with LY294002 (10 μM) for 30 min and then treated with vehicle or OP-1 (200 ng/ml), or IGF-I (25 ng/ml) or the combination of OP-1 (200 ng/ml) and IGF-I (25 ng/ml) for 48 h in the presence of LY294002 (10 μM). The medium was changed every 8 to 16 h. Total AP activity was measured. Values represent the mean ± SE of five independent determinations from three different FRC preparations. The means were normalized to the control, which was defined as 1. $^{*}P < 0.01$ compared with the level of AP activity in control cells. $^{+}P < 0.05$ compared with the level of AP activity in cells that were treated with 200 ng/ml OP-1. $^{**}P < 0.01$ compared with the level of AP activity in cells that were treated with 200 ng/ml OP-1 in combination with 25 ng/ml IGF-I.

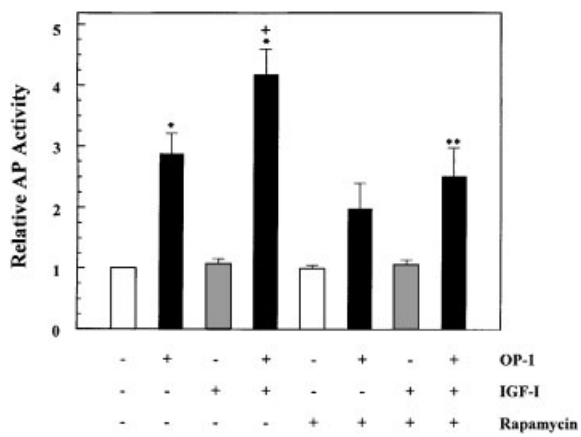

Fig. 6. Effects of rapamycin on the synergistic induction of alkaline phosphatase (AP) activity by Osteogenic Protein-1 (OP-1) and insulin-like growth factor-I (IGF-I) in fetal rat calvaria (FRC) cells. FRC cells were pretreated with rapamycin (109 nM) for 30 min and then treated with vehicle or OP-1 (200 ng/ml), or IGF-I (25 ng/ml) or the combination of OP-1 (200 ng/ml) and IGF-I (25 ng/ml) for 48 h in the presence of rapamycin (109 nM). Total AP activity was measured. Values represent the mean ± SE of five independent determinations from three different FRC preparations. The means were normalized to the control, which was defined as 1. $^{*}P < 0.01$ compared with the level of AP activity in control cells. $^{+}P < 0.05$ compared with the level of AP activity in cells that were treated with 200 ng/ml OP-1. $^{**}P < 0.05$ compared with the level of AP activity in cells that were treated with 200 ng/ml OP-1 in combination with 25 ng/ml IGF-I.

initiated signal transduction. IRS-1 was not detected in FRC cells and its expression was not induced by OP-1 within the 6-h time period examined. IRS-2 was present in FRC cells, and its expression level was not altered by OP-1. The level of expression of the p85α regulatory subunit of the PI 3-kinase was also not altered by OP-1.

Among the ERKs, which represent another major pathway that mediates IGF-I action, ERK-2 was moderately elevated by OP-1 treatment. ERK-1 was significantly increased by OP-1 after 3 h of treatment. Consistent with the present finding is the observation that BMP-2 elevated the ERK protein levels in the pre-osteoblstic cell line, C3H10T1/2 after 10 h of treatment [Lou et al., 2000]. In the contrary, no ERK stimulation was detected up to 3 days following treatment with BMP-2 in C2C12 cells [Vinals et al., 2002].

Our observations that both OP-1 and IGF-I induced ERK phosphorylation indicate that these proteins have a direct effect on ERK activation, and are consistent with several published reports. For example, Hartsough and Mulder [1995] demonstrated that both TGF-β_1 and TGF-β_2 induced rapid ERK phosphorylation in proliferating cultures of epithelial cells. Xu et al. [1996] demonstrated that the Ras/Raf pathway that is known to be upstream of ERK activation, was important for BMP-4 mediated *Xenopus* embryonic development. Palcy and Goltzman [1999] showed an early induction of BMP-2-mediated ERK phosphorylation in chondrogenic and osteoblastic cells lines. In contrast, Lou et al. [2000] reported that ERK phosphorylation was not observed until 10 h after the addition of BMP-2, suggesting an indirect effect of BMP-2 on ERK phosphorylation. Taken together, these findings indicate that the ERK signaling pathway is utilized by the TGF-β super-family including the BMPs.

The cellular effects of the OP-1/BMP-induced rapid ERK phosphorylation are unclear at present. Our data obtained with PD98059, a MEK inhibitor suggests that the ERK signaling pathway is not involved in osteoblast differentiation, as determined by AP activity, a biochemical marker of osteoblastic cell differentiation. Inhibition of ERK phosphorylation did not inhibit the OP-1-induced AP activity or the synergistic effect of OP-1 and IGF-I on AP activity. In contrast, Xiao et al. [2002] reported that MAPK activity is involved in the action of BMP-7 (OP-1) in MC3T3-E1 preosteoblast cells. Lou et al. [2000] also suggested an indirect role for ERK in the BMP-2-induced differentiation of the pre-osteoblastic cell line C3H10T1/2. The differences between these results may be due to the duration of BMP treatment and the cell type. Both Lou et al. and Xiao et al. measured the AP activity in a pre-osteoblastic cell line. The former measured the enzymatic activity 7 days post BMP-2 treatment. We measured the AP activity in primary cultures of FRC cells 48 h post OP-1 treatment. Yue et al. [1999] also suggested a positive regulatory role for ERK in the TGF-β- and the BMP-2-mediated phosphorylation and activation of SMAD-1, a downstream signaling factor of BMP receptors. On the contrary, Kretzschmar et al. [1997] showed that ERK inhibited SMAD-1. Collectively, our current results and the published data indicate that further study is needed to clarify the role of ERK in TGF-β/BMP induced signal transduction and cellular actions.

Our results indicated that the PI 3-kinase activity is important for the OP-1-induced osteoblastic cell differentiation. Inhibition of PI 3-kinase activity using a pharmacological agent, LY294002, completely abrogated the OP-1-induced AP activity. In addition, LY294002 significantly reduced the synergistic effect of OP-1 and IGF-I on AP activity. Of interest is the report of Kozawa et al. [2001] who reported that LY294002 and wortmannin, another inhibitor of PI 3-kinase, significantly inhibited BMP-4-induced VEGF synthesis in osteoblast-like MC3T3-E1 cells. Recently, Ghosh-Choudhury et al. [2002] also reported LY294002 inhibited the induction of AP activity by BMP-2 in 2T3 cells, a mouse calvaria-derived cell line expressing the SV-40 T antigen under the BMP-2 promoter. Furthermore, expression of a dominant-negative PI 3-kinase also eliminated the BMP-2-induced AP activity. Taken together, these findings suggest that PI 3-kinase plays an important role in the BMP-induced cellular effects. At present, it is, however, unclear whether OP-1 directly activates PI 3-kinase.

The current study further showed that treatment of FRC cells with rapamycin resulted in a partial, but significant reduction of the synergistic induction of AP activity by OP-1 and IGF-I, and a near significant reduction in OP-1-induced AP activity. Rapamycin inhibits TOR kinases [Cafferkey et al., 1993; Gingras et al., 2001], proteins that are known to regulate

p70S6 kinase activation [Price et al., 1992; Kunz et al., 1993]. TOR kinases are believed to intercept the PI 3-kinase/p70S6 kinase pathway and have recently been found to be required for PI 3-kinase signaling [Kuruvilla and Schreiber, 1999; Oldham et al., 2000; Zhang et al., 2000; Rohde et al., 2001].

In summary, we demonstrated that PI 3-kinase and p70S6 kinase are involved in the OP-1-induced osteoblast differentiation. OP-1 rapidly induced ERK phosphorylation, even though the physiological effects of this are currently unclear. In addition, we showed that the synergy between OP-1 and IGF-I in inducing osteoblast differentiation is also dependent on PI 3-kinase, and partially dependent on mTOR.

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