Inhibition of PI3K/p70 S6K and p38 MAPK cascades increases osteoblastic differentiation induced by BMP-2

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Abstract Bone morphogenetic proteins (BMPs) transdifferentiate C2C12 cells from the myogenic to the osteogenic lineage. In this work we examine the role of the phosphatidylinositol 3-kinase/p70 S6 kinase (PI3K/p70 S6K) and p38 mitogen-activated protein kinase (p38 MAPK) cascades in the osteogenic effects of BMP-2. BMP-2 stimulated both cascades transiently (maximal at 1 h and decreasing thereafter). In contrast, BMP-2 had no effect on p42/p44 MAPK (Erks) stimulation. We also analyzed the effects of selective inhibitors of these pathways on the expression of osteogenic markers. Inhibitors of p38 MAPK (SB203580) or the PI3K/p70 S6K pathway (Ly294002 and rapamycin) not only fail to block the osteoblast phenotype induced by BMP-2, measured as induction of Cbfa1 expression and transcriptional activity, but also potentiate the effect of BMP-2 on late osteoblast markers, such as alkaline phosphatase activity and osteocalcin expression. These data suggest that, in contrast to their positive effect on myogenic differentiation, PI3K/p70 S6K and p38 MAPK cascades have a negative role in osteoblast differentiation. © 2002 Published by Elsevier Science B.V. on behalf of the Federation of European Biochemical Societies.

Key words: P13K; p70 S6K; p38 MAPK; Osteoblastic differentiation; BMP-2

1. Introduction

Mesenchymal precursors can differentiate to different cellular types, such as adipocytes, myoblasts, fibroblasts or osteoblasts. The fate of a cell depends on mutually exclusive extracellular signalling factors. For example, signals that induce the osteoblast phenotype repress myogenic differentiation in vitro [1,2] and induce bone formation after implantation in intramuscular sites in vivo [3]. The fate of the mesenchymal precursors seems to rely on the expression of 'master' genes, MyoD/Myf5/myogenin for myoblasts, PPARγ2 and C/EBPs for adipocytes or Cbfa1 for osteoblasts [4].

Each differentiation program is ultimately controlled by signals and regulatory pathways that activate a specific set of transcription factors. Among them, bone morphogenetic proteins (BMPs), which belong to the transforming growth factor-β (TGFβ) superfamily of signalling molecules [5] induce osteoblast differentiation in vitro and in vivo, bone regeneration and ectopic bone formation [6]. BMPs also determine crucial steps in embryogenesis, acting as morphogens [5]. BMPs signal through a heterodimeric complex of type I and type II serine/threonine kinase receptors [7,8]. This complex phosphorylates and activates the Smad family of proteins, which transduce the signal to the nucleus [7,9,10]. The BMP-activated Smads are Smad1 and 5, which, upon phosphorylation by the receptor, interact with Smad4 and translocate to the nucleus where they stimulate BMP-induced genes. Other mediators of signals of the TGFβ superfamily members involve kinase cascades such as TAK1 and JNK: TAK functions as a mediator of the MKK6-p38 mitogen-activated protein kinase (MAPK) and MKK7-JNK stimulation and mediates BMP signals in early development in *Xenopus*, whereas JNK mediates activation of fibronectin synthesis from TGFβ receptors [11,12].

In addition to morphogenetic signals that induce the expression of tissue-specific regulatory genes, other signal transduction cascades contribute to cellular commitment. Phosphatidylinositol 3-kinase (PI3K) and p38 MAPK cascades promote myogenic differentiation [13–15]; p38 MAPK activity is also required for chondrogenesis, adipogenesis or neuronal differentiation (reviewed in [16]) and p42/p44 MAPK (or Erks) is required for osteoblastic and neuronal differentiation [1,17–19]. These differentiation programs are initiated by either transcriptional or posttranscriptional activation of cell type-specific transcription factors, as well as the promotion of additional commitment networks. For instance, p38 phosphorylates and activates C/EBPβ during adipogenesis or MEF2C during myogenesis [16], whereas Erks activate Cbfa1 [20]. However, there is no information on the contribution of PI3K/p70 S6K or p38 MAPK signalling cascades to the induction of the osteoblast phenotype. In the present study we show that, in contrast to the myogenic or adipogenic differentiation programs, inhibitors of both cascades do not block, but rather potentiate the osteoblast differentiation of C2C12 cells induced by BMP-2.

2. Materials and methods

2.1. Materials

Human recombinant BMP-2 was obtained from Genetics Institute, SB203580 from Sigma, LY294002 from Alexis and rapamycin from BioMol. Cell culture media, fetal bovine serum (FBS), glutamine and antibiotics were obtained from Gibco BRL. Other reagents were of analytical grade or molecular biology grade and purchased from Sigma or Roche.

2.2. Id183 plasmid

The promoter region of the human *Id1* gene (−1370 to +86) was amplified by polymerase chain reaction (PCR) and subcloned into

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pBluescript vector, sequenced and further subcloned into the promoterless luciferase reporter vector pGL2-basic (pId1lux) (Promega, Madison, WI, USA). Sequences of the primers used for the PCR were 5′-GACAAACTCTTCATCAGAGCTCGCT-3′ (upstream) and 5′-CATGATTCTTGTCGACTGGCTGAAA-3′ (downstream). 5′-Deletions were generated through partial and total *Sma*I digestions using the sites present in the vector and the promoter region. The pId170 reporter construct (−170 to +86) corresponds to the minimal promoter and includes the endogenous TATA box. The 183 bp enhancer fragment identified (corresponding to the −1046 to −863 bp) was subcloned into *Sma*I sites of pId170 generating the pId183 construct.

2.3. Cell culture, transfections and luciferase assay

C2C12 mouse cells were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 20% inactivated FBS, 50 U/ml penicillin and 50 μg/ml streptomycin sulfate. Confluent cells were differentiated in DMEM containing 2% horse serum in the presence or the absence of the indicated concentrations of BMP-2 and the inhibitors. The results obtained were the same independently whether the inhibitors were added once or changed every 24 h.

C2C12 cells were transiently transfected after addition of differentiation medium with the osteoblast-specific region of mouse osteocalcin promoter fused to a luciferase reporter (pOG2-147-Luc) [21] or the Id183 enhancer construct, and a β-galactosidase vector using Fugene (Roche). Luciferase assays were carried out using the Luciferase Assay System (Promega) 16 h (Id183) or 3 days (osteocalcin promoter) after transfection. β-Galactosidase activity was measured using a Luminescent β-galactosidase Detection kit II (Clontech).

2.4. Western blot analysis

Cells were washed twice in cold phosphate-buffered saline (PBS) and lysed in Triton X-100 lysis buffer (50 mM Tris–HCl (pH 7.5), 100 mM NaCl, 50 mM NaF, 5 mM EDTA, 40 mM β-glycerophosphate, 200 μM sodium orthovanadate, 100 μM phenylmethylsulfonyl fluoride, 1 μM pepstatin A, 1 μg/ml leupeptin, 4 μg/ml aprotinin, 1% Triton X-100) for 15 min at 4°C. Insoluble material was removed by centrifugation at 12 000×*g* for 5 min at 4°. Proteins were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) and electrophoretically transferred to Immobilon-P membranes (Millipore) in 25 mM Tris–HCl, 0.19 M glycine and 20% ethanol. Membranes were blocked in TBS (50 mM Tris, pH 7.4, 150 mM NaCl) containing 5% non-fat dry milk for 1 h at 37°C. The blots were incubated with polyclonal anti-phospho-(Ser473)-Akt antibody (New England Biolabs), polyclonal anti-Akt antibody (New England Biolabs), polyclonal anti-p42 MAPK [22], monoclonal anti-phospho-Erk1/2 antibody (Sigma), polyclonal anti-phospho-p70 S6K antibody (New England Biolabs), polyclonal anti-phospho-p38 MAPK antibody (Promega), polyclonal anti-p38 MAPK antibody (Upstate Biotechnology), polyclonal anti-$p21^{Waf1/Cip1}$ antibody (Santa Cruz), polyclonal anti-phospho-Smad1 (Upstate Biotechnology) and polyclonal anti-Cbfa1 antibody (Santa Cruz) in blocking solution overnight at 4°C. After washing in TBS, 0.1% Tween 20, blots were incubated with anti-rabbit Ig (Amersham Pharmacia), anti-mouse Ig (Amersham Pharmacia) or anti-goat Ig (Dako) horseradish peroxidase linked antibodies in blocking solution for 1 h and developed with an enhanced chemiluminescence system (Amersham Pharmacia).

2.5. Northern blot

Total RNA from cells was extracted using the phenol/chloroform method [23]. 20 μg of RNA were denatured at 65°C in the presence of formamide, formaldehyde and ethidium bromide. RNA was separated on a 1.5% agarose/formaldehyde gel and blotted on Hybond N+ filters (Amersham Pharmacia). Blots were hybridized to the mouse osteocalcin cDNA (a generous gift from Dr. G. Karsenty, Baylor College of Medicine, Houston, TX, USA) or rat GAPDH cDNA labelled with [α-^{32}P]dCTP (Amersham Pharmacia), and incubated overnight at 42°C in 50% formamide, 5× Denhardt's (1× Denhardt's is 0.02% Ficoll, 0.02% polyvinylpyrrolidone and 0.02% bovine serum albumin) 0.1% SDS, 5× SSPE (1× SSPE is 10 mM NaH_2PO_4, pH 7.4, 150 mM NaCl and 1 mM EDTA), 5% dextran sulfate, and 200 μg/ml denatured salmon sperm DNA. After hybridization, membranes were washed in 0.1× SSC (1× SSC is 15 mM sodium citrate, pH 7 and 150 mM NaCl)–0.1% SDS at 50°C for 50 min. The blots were exposed to Kodak films.

2.6. Alkaline phosphatase activity

Cells were washed twice in PBS and lysed on 20 mM Tris–HCl, pH 8, 150 mM NaCl, 0.2% NP40 and 10% glycerol. Cell lysates (25 μg) were incubated in a buffer containing 0.1 M glycylglycine, 10 mM $MgCl_2$, 1 mM $ZnSO_4$, 10 mM *p*-nitrophenyl phosphate, pH 9.5 at 37°C for 2 h. The reaction was stopped with 0.5 N NaOH and absorbance was measured at 405 nm.

3. Results

3.1. Activation of different protein kinase cascades during BMP-2-induced osteoblast differentiation of C2C12 cells

As previously described, C2C12 cells with a limited supply of growth factors (2% horse serum) differentiate into myoblasts, which later fuse into multinucleated myotubes (Fig. 2). Treatment with BMP-2 completely blocked myoblast differentiation (Fig. 2, [24,25]). We analyzed the activation of p70 S6K, Akt, p38 and p42/p44 MAPKs during differentiation of C2C12 cells in the presence or the absence of BMP-2. Depletion of growth factors caused a progressive drop in PI3K (measured by phosphorylation of Akt, a downstream target), p70 S6K and p42/p44 MAPK activities (Fig. 1), until

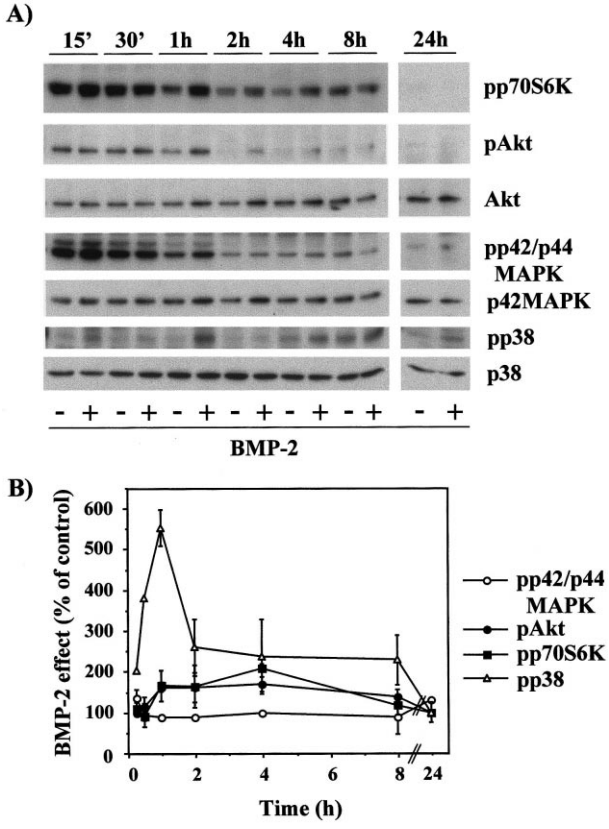

Fig. 1. Effect of BMP-2 stimulation on p70 S6K, Akt, p42/p44 MAPK and p38 MAPK activities. A: Confluent C2C12 cells were incubated (for the periods of time indicated) in DMEM supplemented with 2% horse serum in the absence or in the presence of 10 nM BMP-2. Cells were lysed and Western blot was performed using anti-active p70 S6K (pp70 S6K), anti-active Akt (pAkt), total Akt (Akt), anti-active p42/p44 MAPK (pp42/p44 MAPK), total p42 MAPK (p42 MAPK), anti-active p38 MAPK (pp38) and total p38 MAPK (p38) antibodies. A representative Western blot is shown. B: Quantification of data shown in (A). Results were normalized by the level of p42 MAPK. Values represent the mean of three independent experiments and are expressed relative to their respective control in the absence of BMP-2.

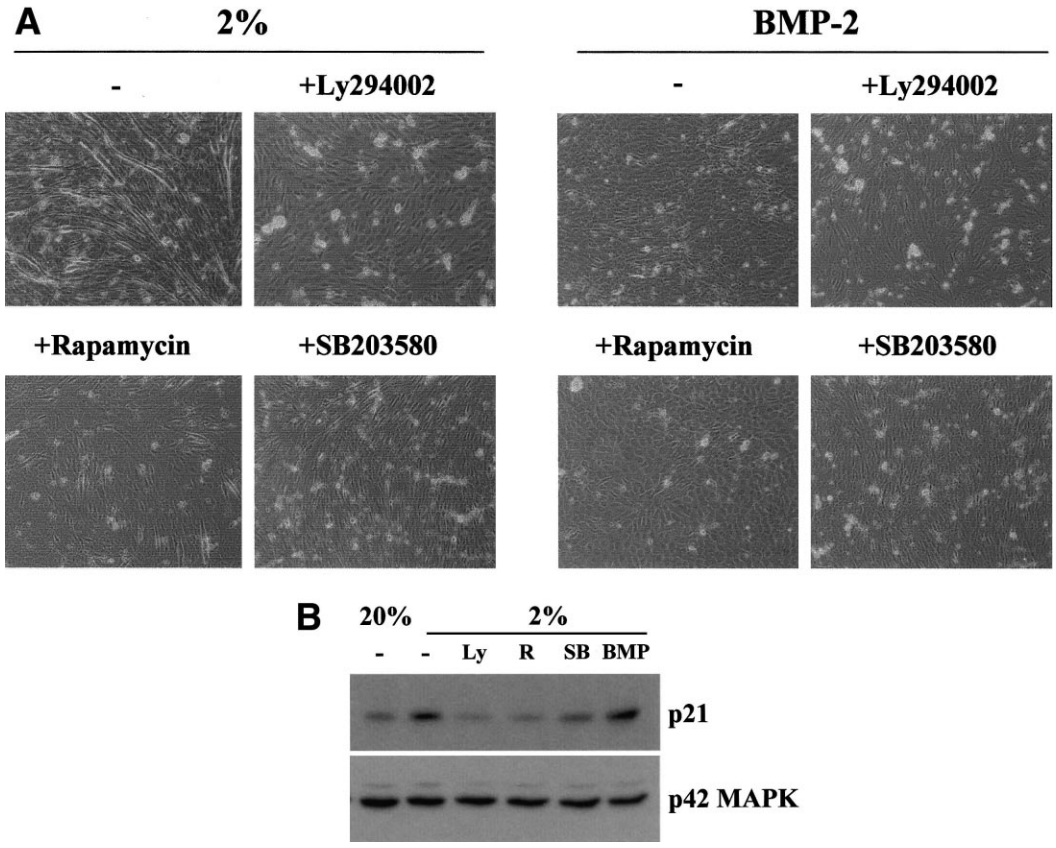

Fig. 2. BMP-2, Ly294002, rapamycin and SB203580 block skeletal muscle differentiation. A: Confluent C2C12 cells were depleted of growth factors and incubated in a medium containing 2% horse serum for 3 days in the absence or the presence of 2 nM BMP-2, 15 μM Ly294002, 10 nM rapamycin or 15 μM SB203580. Cells were examined by phase-contrast microscopy and photographed. Magnification: 100×. B: Confluent C2C12 cells were depleted or not (20%) of growth factors and incubated in a medium containing 2% horse serum for 3 days in the absence (2%) or the presence of 2 nM BMP-2, 15 μM Ly294002 (Ly), 10 nM rapamycin (R) or 15 μM SB203580 (SB). After lysis 50 μg of protein was separated on an SDS–12% polyacrylamide gel, and $p21^{Waf1/Cip1}$ or p42 MAPK (as protein loading control) were immunodetected with specific antibodies. A representative Western blot is shown.

the complete inactivation of these signaling pathways after 24 h. In contrast, p38 MAPK activity increased slightly after 4 h in differentiation medium, peaked at 8 h and returned to basal levels after 24 h. Addition of BMP-2 transiently stimulated p38 MAPK at 15 min and activation was maximal at 1 h. BMP-2 also stimulated Akt and p70 S6K, but only after incubation for at least 1 h. The stimulation was maintained until 4 h and progressively normalized after 8 h. No effect of BMP-2 was observed thereafter for up to 3 days, or on p42/p44 MAPK (Fig. 1) or JNK activities (data not shown).

3.2. Effect of inhibitors of PI3K/p70 S6K and p38 MAPK cascades on the osteoblast differentiation of C2C12 cells induced by BMP-2

To examine the role of PI3K, p70 S6K and p38 MAPK activities in the induction of osteogenic differentiation by BMP-2, C2C12 cells were incubated in differentiation medium in the presence or the absence of BMP-2 with either 15 μM Ly294002, a specific inhibitor of PI3K activity, 10 nM rapamycin, a specific inhibitor of p70 S6K activity, or 10 μM SB203580, a specific inhibitor of p38 MAPK. As described before [14], all these inhibitors blocked the induction of the skeletal muscle phenotype visualized as myotube formation (Fig. 2A) or the induction of the $p21^{Waf1/Cip1}$ (Fig. 2B), whereas the presence of concentrations as low as 2 nM BMP-2

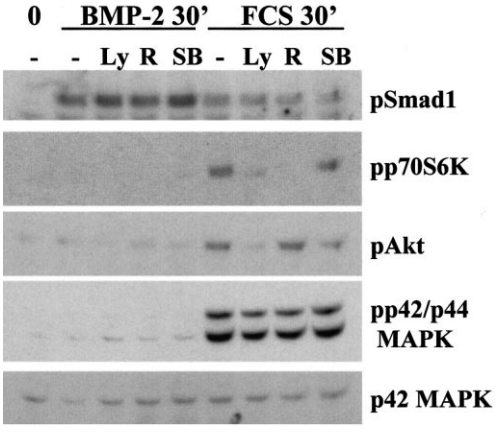

Fig. 3. PI3K, p70 S6K or p38 MAPK inhibition does not affect BMP-2 signaling cascade. Quiescent C2C12 cells were preincubated for 15 min in the absence (−) or the presence of 15 μM Ly294002 (Ly), 10 nM rapamycin (R) or 15 μM SB203580 (SB). After this time, cells were stimulated for 30 min with 2 nM BMP-2 or 10% FCS or were left unstimulated (0). Cells were lysed, and phospho-Smad1 (pSmad1), phospho-p70 S6K (pp70 S6K), phospho-Akt (pAkt), phospho-p42/p44 MAPK (pp42/p44 MAPK) and p42 MAPK were immunodetected as described. A Western blot representative of three different experiments is shown.

completely blocked myotube formation, with no differences in the presence of the various inhibitors.

In order to determine whether the inhibition of these signaling pathways influences the BMP-2 signal transduction cascade, we measured the effect of treatment with Ly294002, rapamycin and SB203580 on the stimulation by BMP-2 of the phosphorylation of Smad1. The addition of all these inhibitors did not alter the BMP-2-induced phosphorylation of Smad1 (Fig. 3), whereas preincubation with Ly294002 specifically prevented PI3K activation by fetal calf serum (FCS; measured by the phosphorylation state of two downstream targets, Akt and p70 S6K) and incubation with rapamycin blocked the p70 S6K stimulation (Fig. 3). The specificity of this inhibition was confirmed by the absence of p42/p44 MAPK inhibition in response to Ly294002 or rapamycin. Moreover, we have measured the effect of all these inhibitors on the stimulation by BMP-2 of the promoter of Id1, an immediate early gene induced by BMP-2. We have identified a BMP-2 responsive region in the Id1 promoter which is sufficient to confer BMP-2 responsiveness (Teresa López-Rovira et al., JBC manuscript in press). The addition of all these inhibitors did not modify the transcriptional responses on Id promoter by BMP-2 (3.6 ± 0.7-fold induction by BMP-2 alone, 4.1 ± 1.1-fold induction by BMP-2 in the presence of 15 μM Ly294002, 4.6 ± 1.2-fold in the presence of 10 mM rapamycin and 4.8 ± 0.7-fold in the presence of 10 μM SB203580). All these results indicate a lack of effect of the p38 MAPK and PI3K/p70 S6K pathways on the signaling cascade induced by BMP-2.

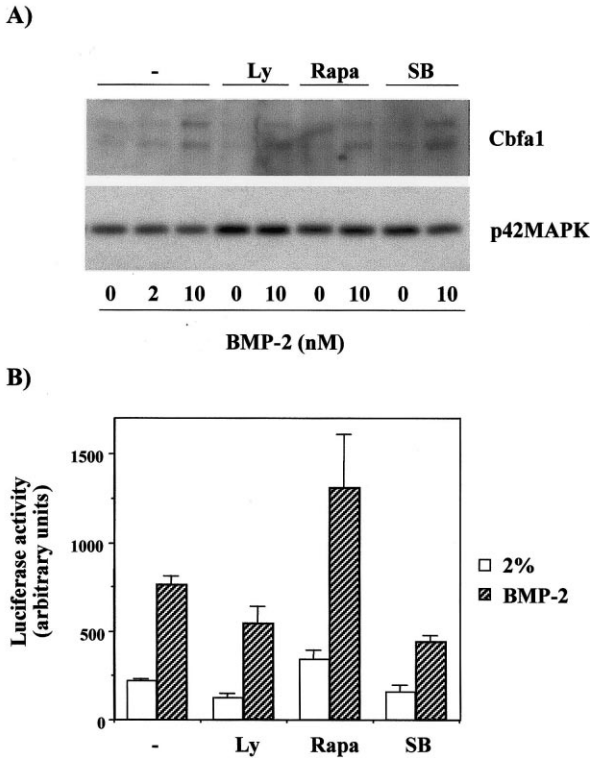

Fig. 4. PI3K, p70 S6K or p38 MAPK inhibition does not affect Cbfa1 induction and transcriptional activity. A: Confluent C2C12 cells were allowed to differentiate in the absence (0) or in the presence of 2 or 10 nM BMP-2 alone or in the presence of 15 μM Ly294002 (Ly), 10 nM rapamycin (Rapa) or 15 μM SB203580 (SB) for three days. After lysis 30 μg of protein was separated on an SDS–10% polyacrylamide gel, and Cbfa1 or p42 MAPK (as protein loading control) were immunodetected with specific antibodies. A representative Western blot is shown. B: C2C12 cells depleted of growth factors were transiently transfected with osteocalcin-luciferase reporter and β-galactosidase vector, and incubated for 3 days in the absence (2%) or the presence of 2 nM BMP-2 alone or in the presence of 15 μM Ly294002 (Ly), 10 nM rapamycin (Rapa) or 15 μM SB203580 (SB). After this time cells were harvested and luciferase and β-galactosidase activities were measured. The results are the mean ± S.E.M. of three independent experiments and are expressed as relative luciferase/β-galactosidase activities.

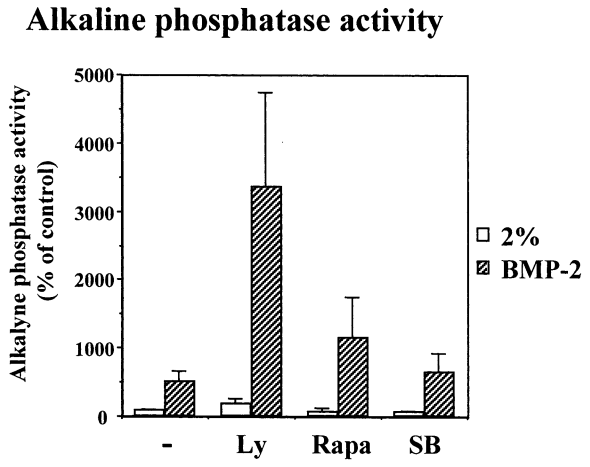

Fig. 5. PI3K, p70 S6K or p38 MAPK inhibition does not block but stimulates alkaline phosphatase activity. Confluent C2C12 cells were depleted of growth factors and incubated in a medium containing 2% horse serum for 3 days in the absence (2%) or the presence of 2 nM BMP-2, 15 μM Ly294002 (Ly), 10 nM rapamycin (Rapa) or 15 μM SB203580 (SB). After this time cells were lysed and alkaline phosphatase was determined as described in Section 2. Data are the mean ± S.E.M. of three independent experiments.

Next, we have determined the requirement of these signalling pathways for osteoblast differentiation, we first studied the induction of an earliest marker of the osteoblast phenotype, the transcription factor Cbfa1 [26]. This marker, immunodetected as a double band [27], was present at low levels on C2C12 cells after 3 days in differentiation media ([4], Fig. 4A). Incubation with BMP-2 induced Cbfa1 at 24 h (data not shown) and at 3 days (Fig. 4A). More importantly, Cbfa1 was similarly induced by BMP-2 in the presence of the different inhibitors, Ly294002, rapamycin and SB203580. Since p42/p44 MAPK activity phosphorylates and regulates Cbfa1 activity [20] we examined whether Cbfa1 transcriptional activity was affected by PI3K/p70 S6K or p38 MAPK inhibition. With this objective we measured the activity of a reporter construct containing the minimal osteoblast-specific enhancer of the osteocalcin promoter that contains a Cbfa1-responsive region [21,26]. As shown in Fig. 4B, after 3 days in differentiation media, BMP-2 induced a 2.5-fold increase in the osteocalcin reporter activity. Addition of the different inhibitors did not significantly affect the basal reporter activity or the BMP-2 fold induction (2.5-fold increase without inhibitors, 3.1-fold in the presence of Ly294002, 2.8-fold in the presence of rapamycin, and 2.7-fold in the presence of SB203580). These results indicate that, in contrast with their requirement for skeletal muscle differentiation, PI3K/p70 S6K cascade and p38 MAPK activation are not essential for BMP-2 induction of Cbfa1 gene expression and transcriptional activity.

Finally, we analyzed the effects of the inhibitors of the

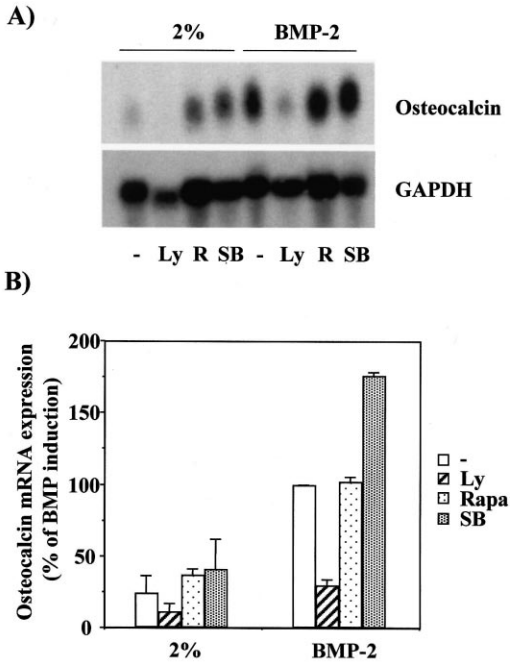

Fig. 6. PI3K, p70 S6K or p38 MAPK inhibition does not block osteocalcin mRNA. A: Confluent C2C12 cells were depleted of growth factors and incubated in a medium containing 2% horse serum for 6 days in the absence (2%) or the presence of 2 nM BMP-2, 15 μM Ly294002 (Ly), 10 nM rapamycin (R) or 15 μM SB203580 (SB). After this time total RNA was obtained from the different experimental conditions and 20 μg was load on gels. After blotting, osteocalcin and GAPDH were detected by hybridization using specific probes. A representative autoradiogram is shown. B: Quantification of data shown in (A). Results were normalized by the level of GAPDH. Values represent the mean of two independent experiments and are expressed relative to their respective BMP-2-induced effect.

PI3K/p70 S6K and p38 MAPK cascades on markers of osteoblast terminal differentiation. First, treatment with BMP-2 induced a five-fold increase in alkaline phosphatase activity after 3 days (Fig. 5). Control treatments with rapamycin or SB203580 did not significantly affect the basal alkaline phosphatase activity, whereas Ly294002 slightly increased basal activity. More importantly, the inhibitors not only failed to block the BMP-stimulation but rather potentiated its effect, with a 17-fold increase in the presence of Ly294002, a 13-fold increase in the presence of rapamycin, and a nine-fold increase in the presence of SB203580. Finally, we studied the expression of the later osteoblast marker osteocalcin, by measuring its mRNA by northern analysis. As shown in Fig. 6, treatment with BMP-2 for 6 days induced osteocalcin mRNA. Incubation of cells in the presence of Ly294002 for 6 days had a deleterious effect on cell viability, with a strong decrease in cell number and mRNA levels, as shown by basal GAPDH expression (Fig. 6). However in the presence of BMP-2 and Ly294002, osteocalcin mRNA was similarly induced to control cells. Incubation of C2C12 cells in 2% horse serum in the presence of rapamycin or SB203580 increased the basal levels of osteocalcin, while the capacity of BMP-2 to induce osteocalcin mRNA was also maintained.

4. Discussion

Although activation of PI3K/p70 S6K and MAPK signalling cascades has been associated with many different physiological events, there is little evidence on the contribution of these pathways to osteoblast differentiation. Here we found that BMP-2 activates both p38 MAPK and PI3K cascade in C2C12 cells. Moreover, we have shown that inhibition of PI3K/p70 S6K and p38 MAPK, two cascades that participate in skeletal muscle and chondrocyte differentiation, are not required on the osteoblastic differentiation program induced by BMP-2. Finally, repression of these cascades potentiates the effect of BMP-2 inducing two late markers of osteoblast differentiation, alkaline phosphatase and osteocalcin.

Activation of p38 MAPK by BMP-2 has also been observed in PC12 cells during neuronal differentiation [28], in the mouse chondrogenic cell line ATDC5 [29] and in mouse hybridoma HM60 cells [30]. This early stimulation of p38 is required for some transcriptional responses to TGFβ superfamily members [28,31,32]. These effects might be related to the activation of TAK1, a MAPKKK member that was reported to be activated as soon as 15 min after TGFβ or BMP-2 addition, and which provides a link between signalling by TGFβ superfamily members and JNK and p38 MAPK activation [33,34]. In contrast, no direct effect of BMP-2 on the PI3K/p70 S6K cascade has been described. The latency observed for the BMP-2 effect on this cascade by us and others [35] suggests an indirect effect of BMP-2 signalling that could be mediated by activation of a regulatory intracellular network or by release of an unknown autocrine factor.

In contrast to the stimulatory effect of BMP-2 on p38 MAPK and PI3K/p70 S6K activities, blockage of these signals by specific inhibitors does not affect the BMP-2-dependent induction of the osteoblast phenotype. Surprisingly, the lack of requirement of p38 MAPK and PI3K/p70 S6K for osteogenesis contrasts with their effects on other mesenchymal differentiation programs, such as myogenesis or chondrogenesis. Indeed, inhibition of p38 MAPK blocks chondrogenesis in micromass cultures of mesenchymal cells or in the ATDC5 chondrogenic cell line [29,36], and also inhibits myogenesis and myoblast fusion of C2C12 or L8 cells [14,37]. It has also been shown that inhibition of PI3K blocks myogenic differentiation [13,38] and that the PI3K-downstream kinase Akt participates in this promyogenic effect [39,40]. Both signaling cascades, p38 MAPK and PI3K, seem to be independent but necessary for the final muscle phenotype [41]. Confirming all these data, SB203580, Ly294002 and rapamycin all blocked myogenic differentiation in our culture of C2C12 cells after growth factor depletion (Fig. 2). However, at the same dose which completely inhibits their specific activities, these inhibitors not only failed to block BMP-2-induced increases in Cbfa1 expression and transcriptional activity but even potentiated the expression of late differentiation markers. A direct inhibitory effect of the p38 MAPK or PI3K/p70 S6K on BMP-2 signalling pathway is unlikely, since addition of specific inhibitors did not alter BMP-2-induced signaling or transcriptional responses, and inhibition of p38 MAPK blocks neuronal or chondrogenic differentiation programs induced by BMPs [28,29].

Previous evidence supports the hypothesis that other signals, apart from Cbfa1, are necessary to final osteoblast differentiation, and that they would be targets of negative regulation by p38 MAPK and PI3K/p70 S6K. For instance, the same effect has been shown in rat osteoblast-like osteosarcoma (ROS 17/2.8) cells, where rapamycin alone induces osteo-

calcin expression and alkaline phosphatase activity, and synergizes with $1,25(OH)_2D_3$ to induce osteoblast differentiation [42]. In addition, whereas Cbfa1 seems to play a key role in osteoblast determination of mesenchymal cells, expression of this transcription factor alone fails to induce the final osteoblast phenotype (osteocalcin or alkaline phosphatase activity) [4]. This ability of different MAPK pathways to switch the fate of a pluripotent mesenchymal cell population has also been observed after inhibition of p42/p44 MAPK cascade, switching from osteogenesis to adipogenesis [19]. These data indicate that commitment to a mesenchymal differentiation program depends not only on the expression of known transcriptional 'master' genes but also on a delicate equilibrium between different signaling cascades.

Different types of cellular stress, such as mechanical loading and hyperosmolarity generated by calcification of bone extracellular matrix, cause osteoblasts and osteoclasts to induce bone remodeling. This type of stimulus has been shown to activate p38 MAPK, inhibit p70 S6K [16,43] and to influence bone remodeling and matrix deposition both in organ cultures and in vivo [44,45]. In addition, the use of immunosuppressants after organ transplantation, such as cyclosporin A or FK-506, leads to osteoporosis, whereas rapamycin is bone-sparing [46]. In this respect, inhibition of either p38 MAPK or PI3K/p70S6K blocks the differentiation and function of osteoclasts [47–49] whereas in this study we found that they potentiate the osteoblast function. Taken together, these lines of evidence suggest a crucial role of p38 MAPK and PI3K/p70S6K in balancing the differentiation and function of both the osteoblast and osteoclast lineages, which is relevant to our understanding of the mechanisms of bone remodeling.

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