Protein kinase signalling pathways involved in the up-regulation of the rat $\alpha 1(I)$ collagen gene by transforming growth factor β_1 and bone morphogenetic protein 2 in osteoblastic cells

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Transforming growth factor β (TGF β) family members are known for their important role in bone physiology. TGF β_1 and, to a smaller extent, bone morphogenetic protein 2 (BMP-2) have been reported to regulate the gene expression of different osteoblast markers *in vitro*. However, little is known about the molecular mechanisms involved in these actions. Here we report that BMP-2, like TGF β_1 , up-regulated $\alpha 1(I)$ collagen mRNA expression in ROS 17/2.8 osteoblastic cells. This was mediated through an increase in the transcriptional rate of the gene rather than through the stabilization of $\alpha 1(I)$ collagen mRNA, and required new protein synthesis. In addition, TGF β_1 - and BMP-2-induced increases in $\alpha 1(I)$ collagen mRNA levels were both dependent on protein kinase C and protein tyrosine kinase activities. Furthermore, the mitogen-activated protein kinase (MAPK) [MAPK/extracellular signal-regulated protein kinase kinase 1/extracellular signal-regulated protein kinase

(MEK-1/ERK)] pathway participated in the up-regulation of $\alpha 1(I)$ collagen gene expression by TGF β_1 and BMP-2. In response to either TGF β_1 or BMP-2, the stimulation of $\alpha 1(I)$ collagen mRNA levels was paralleled by an early increase in extracellular signal-regulated kinase protein activity. Moreover, the effects of both TGF β_1 and BMP-2 on $\alpha 1(I)$ collagen gene expression were markedly decreased in transfected ROS 17/2.8 cells expressing a dominant-negative MEK-1. Our findings therefore show that TGF β_1 and BMP-2, which signal through discrete cell-surface receptors, are able to trigger analogous, if not identical, protein-phosphorylation-transducing cascades leading to comparable actions on the transcription of the $\alpha 1(I)$ collagen gene in osteoblastic cells.

Key words: mitogen-activated protein kinase, mRNA expression, protein kinase C, ROS 17/2.8 cells.

INTRODUCTION

Members of the transforming growth factor β (TGF β) family, TGF β and bone morphogenetic proteins (BMPs), have a major role in bone formation. Over the past decade, several studies have demonstrated TGF β_1 and BMP-2 osteogenic properties *in vivo* as well as their ability to modulate osteoblast cell proliferation and differentiation *in vitro* [1]. In cellular models, TGF β_1 has been reported to regulate the expression of specific genes such as osteocalcin, osteopontin, osteonectin, type I collagen, alkaline phosphatase [2] and the parathyroid hormone/parathyroid-hormone-related peptide ('PTH/PTHrP') receptor [3]. The effects of BMP-2 on osteoblastic cells have been less intensively investigated. However, BMP-2 has also been found to regulate the expression of alkaline phosphatase, the production of PTH-dependent cAMP and the synthesis of collagen [4–6]. Comparative studies have revealed that TGF β_1 and BMP-2 can exert similar or opposite effects on osteoblast functions, depending on the studied cellular response and the cell line [5]. Although TGF β_1 and BMP-2 are important factors for the regulation of the osteoblast phenotype, little is known about the molecular mechanisms involved in these actions.

TGF β_1 and BMP-2 signal through their interaction with specific serine/threonine kinase cell surface receptors. For both factors, two classes of receptor (type I and type II) have been identified as signal-transducing receptors [7]. The type I and type II TGF β_1 receptors form an oligomeric complex to bind their ligand co-operatively and transduce signals. BMP-2 type I and

type II receptors are also associated in a complex but the co-operativity of the two receptor species towards the binding of ligand is different from that of the type-I–type-II TGF β_1 complex. Type I and type II receptors for TGF β_1 have been characterized in osteoblastic cells [8].

Other key components for the biological activities of TGF β_1 and BMP-2 are signal transduction elements acting downstream of the serine/threonine kinase receptor. Findings in non-osteoblastic cells suggest that phospholipase C is involved in the effects of TGF β_1 on cell proliferation [9] and gene expression [10]. Protein kinase C (PKC)-dependent activities have been also found to participate in TGF β_1 -induced gene expression [10,11]. In addition, components of the mitogen-activated protein kinase (MAPK) cascade have been shown to contribute to the regulation of gene transcription by TGF β_1 in a variety of cell types [11–13]. Recently the SMAD family (for nomenclature see reference [13a]) of signal transducers has been discovered and implicated in cell signalling by members of the TGF β superfamily [14]. The intracellular components of TGF β_1 and BMP-2 signalling in osteoblastic cells are as yet largely unknown. However, a novel member of the MAPK kinase family, TGF β -activated kinase 1 ('TAK1'), has been found to activate the stress-activated protein kinase ('SAPK') pathway in response to TGF β in MC 3T3-E1 rat osteoblastic cells [15].

In the present study we compared the effects of TGF β_1 and BMP-2 on the gene expression of $\alpha 1(I)$ collagen in the rat osteoblastic cell line ROS 17/2.8 and examined the transduction events, downstream of the receptor, involved in these actions.

Abbreviations used: AP-1, activator protein 1; BMP, bone morphogenetic protein; DRB, 5,6-dichloro-1- β -D-ribofuranosylbenzimidazole; ERK, extracellular signal-regulated protein kinase; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; MAPK, mitogen-activated protein kinase; MBP, myelin basic protein; MEK, MAPK/ERK kinase; PAI-1, plasminogen activator inhibitor 1; PKC, protein kinase C; PTK, protein tyrosine kinase; TGF β , transforming growth factor β .

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We show that BMP-2, like $TGF\beta_1$, stimulates $\alpha 1(I)$ collagen mRNA transcription in this cell line and demonstrate that in both cases PKC- and protein tyrosine kinase (PTK)-dependent activities participate in this action. Furthermore, we demonstrate the participation of the MAPK [MAPK/extracellular signal-regulated protein kinase kinase 1/extracellular signal-regulated protein kinase (MEK-1/ERK)] pathway in the up-regulation of $\alpha 1(I)$ collagen gene expression by $TGF\beta_1$ and BMP-2.

EXPERIMENTAL

Reagents

Human recombinant $TGF\beta_1$ was purchased from Life Technologies (Gaithersburg, MD, U.S.A.). Human recombinant BMP-2 was kindly provided by the Genetics Institute (Cambridge, MA, U.S.A.). Calphostin C, chelerythrine chloride, staurosporine, genistein and 5,6-dichloro-1- β -D-ribofuranosylbenzimidazole (DRB) were all purchased from Biomol (Plymouth Meeting, PA, U.S.A.). Cycloheximide and actinomycin D were from Sigma (St. Louis, MO, U.S.A.).

Cell culture

Rat osteosarcoma ROS 17/2.8 cells [16] were cultured at 37 °C in Dulbecco's modified Eagle's medium/F12 medium (Life Technologies) supplemented with 10 mM Hepes and 10% (v/v) fetal bovine serum (WISENT, St. Bruno, Quebec, Canada), under air/CO₂ (19:1) in a humidified incubator. The cells were routinely subcultured every 3 days. For all experiments, cells were seeded at 1.5×10^4 cells/cm² in 60 mm culture plates in medium containing 5% (v/v) fetal bovine serum, then grown for 48 h. After removal of the culture medium, the cell layer was rinsed once with serum-free medium and fed with fresh medium containing 2% (v/v) fetal bovine serum, with or without the indicated reagent(s) or the corresponding vehicle. The viability of the ROS 17/2.8 cells in the presence of the different kinase inhibitors was assessed after 48 h (or 24 h) of incubation at the highest dose by the Trypan Blue exclusion method. Under these experimental conditions the percentage of viable cells was more than 94.6% in all cases.

Northern blot analysis

Total RNA was isolated with the Trizol reagent in accordance with the protocol of the manufacturer (Life Technologies), fractionated on 1% (w/v) agarose/formaldehyde gel (10 μ g per lane) and transferred to a nylon membrane (Hybond N+; Amersham, Oakville, Ontario, Canada). Filters were pre-hybridized for 2 h at 65 °C in hybridization buffer [HB; 0.3 M Na₂HPO₄/0.2 M NaH₂PO₄/7% (w/v) SDS/1 mM EDTA/1% (w/v) BSA (pH 7.0)], then hybridized overnight in the same buffer containing 10⁶ c.p.m./ml specific cDNA. Rat type I collagen ($\alpha 1(I)$) cDNA [17] was a gift from Dr. D. Rowe. Filters were washed twice for 15 min in 2 $\times$ standard saline citrate (SSC)/0.5% SDS at 65 °C, then autoradiographed. As a control for loading, filters were probed in parallel with human glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA [18]. Signals were quantified by scanning densitometry and normalized against GAPDH mRNA values. Student's *t* test was used to determine statistical significance.

Transcription assay *in vitro*

Nuclei were isolated and transcription was pursued *in vitro* in the presence of [α -³²P]UTP by the method of Fei and Drake [19]. The

radiolabelled transcripts were purified with the Trizol reagent (see above). $\alpha 1(I)$ collagen and GAPDH cDNA species (see above) were immobilized on a nylon membrane (see above). Filters prehybridized overnight at 65 °C in HB were hybridized for 72 h at 65 °C in the same buffer containing the radiolabelled transcripts. After being washed (twice for 15 min each in 2 $\times$ SSC/0.5% SDS at 65 °C), filters were subjected to autoradiography. Signals were quantified by scanning densitometry.

Immune complex kinase assay

The cells were washed twice in ice-cold PBS, scraped off plates in cell lysis buffer [20 mM Tris/HCl (pH 7.5)/150 mM NaCl/1% (v/v) Nonidet P40/10% (v/v) glycerol/1 mM PMSF/1 μ g/ml leupeptin/1 μ g/ml pepstatin/1 mM sodium orthovanadate/1 mM sodium metavanadate], then sonicated for 20 s. Lysates were cleared by centrifugation at 12000 *g* for 15 min and protein concentrations were determined with a bicinchoninic acid assay (Micro BCA; Pierce, Rockford, IL, U.S.A.). Equal amounts of protein were rotated overnight with 1 μ g of anti-ERK2 (C14; Santa Cruz Biotechnology, Santa Cruz, CA, U.S.A.). The anti-ERK2 antibodies cross-react with the ERK1 form. After binding to Protein A-Sepharose (Pharmacia Biotech, Uppsala, Sweden), the immune complexes were washed three times with cell lysis buffer and twice with kinase buffer [50 mM Tris/HCl (pH 8.0)/25 mM MgCl₂/1 mM dithiothreitol/0.5 mM EGTA/10% (v/v) glycerol] before being resuspended in 30 μ l of kinase buffer reaction mixture [kinase buffer containing 20 μ M ATP, 1 μ Ci of [γ -³²P]ATP and 0.5 mg/ml myelin basic protein (MBP; Sigma)]. The phosphorylation reaction was stopped after 30 min at 30 °C by the addition of 10 μ l of 4 $\times$ SDS sample buffer [15% (v/v) glycerol, 125 mM Tris/HCl (pH 6.8)/5 mM EDTA/2% (w/v) SDS/0.1% Bromophenol Blue/1% (v/v) 2-mercaptoethanol]. SDS/PAGE analysis of the samples was followed by autoradiography. The incorporation of ³²P into MBP was analysed by scanning densitometry.

Cell transfections

A cDNA encoding the dominant-negative form of MEK-1 was created by the mutation of lysine-97 to alanine, as described previously [20]. This mutation was performed by PCR with wild-type MEK-1 cDNA as a template and the oligonucleotides 5'-GGCCAGAGCGCTAATTCATCTGG-3' as sense primer and 5'-CCAGATGAATTAGCGCTCTGGCC-3' as anti-sense primer (changes from the original sequence are underlined). The mutated cDNA (MEK-1_{Ala-97}) was then subcloned into *Bam*HI and *Xho*I sites of the pCDNA3 mammalian expression vector (InVitrogen). The mutation was sequenced to ensure its correct composition. MEK-1_{Ala-97} expression vector (pCMEK-1_{Ala-97}), or pCDNA3 empty vector (pC) as a control, was transfected into ROS 17/2.8 cells with the lipofectin reagent (Life Technologies). Stable clones were selected in culture medium containing 500 μ g/ml G418. Resistant clones were then tested for the expression of MEK-1_{Ala-97} or neomycin by Northern blotting. Two positive clones either expressing MEK-1_{Ala-97} (pCMEK-1_{Ala-97}-1 and pCMEK-1_{Ala-97}-2) or containing the empty vector (pC-1 and pC-2) were used for further experiments.

RESULTS

Regulation of $\alpha 1(I)$ collagen mRNA expression by $TGF\beta_1$ and BMP-2

To compare the activities of $TGF\beta_1$ and BMP-2 on gene expression in osteoblastic cells, we decided to examine whether

Figure 1 Effects of TGF β_1 and BMP-2 on $\alpha 1(I)$ collagen (COL I) mRNA levels

Cells were treated for 24 or 48 h with increasing doses of TGF β_1 (T) or BMP-2 (B). Total RNA was isolated at each time point and subjected to Northern blot analysis, as described in the Experimental section. A representative autoradiogram from four independent experiments is shown.

Figure 2 Effects of cycloheximide and actinomycin D on TGF β_1 - and BMP2-induced increases in $\alpha 1(I)$ collagen (COL I) mRNA levels

Cells were treated or not (control, C) with TGF β_1 (T, 5 ng/ml) or BMP2 (B, 50 ng/ml) for 24 h, in the presence of vehicle (ethanol) or (A) cycloheximide (CX, 0.5 μ g/ml) or (B) actinomycin D (AD, 3 ng/ml). Northern blot analysis was performed on total RNA, as described in the Experimental section. A representative autoradiogram from two independent experiments is shown.

or not BMP-2 could mimic the effect of TGF β_1 on the expression of $\alpha 1(I)$ collagen mRNA. TGF β_1 has previously been described to increase the levels of $\alpha 1(I)$ collagen mRNA in ROS 17/2.8 cells [2]. The cells were treated for 24 and 48 h with increasing doses of TGF β_1 or BMP-2; the isolated total RNA was subjected to Northern blot analysis. As shown in Figure 1, the steady-state

level of $\alpha 1(I)$ collagen mRNA was increased in a dose- and time-dependent manner by both TGF β_1 and BMP-2. For each of the two factors, the effect was detectable after 24 h of incubation and lasted for at least 48 h of treatment. In both cases no effect was observed before 24 h (results not shown). Interestingly, BMP-2 produced a greater increase in $\alpha 1(I)$ collagen mRNA expression than did TGF β_1 . Thus, after 48 h of incubation, the maximal dose of BMP-2 used (50 ng/ml) led to a maximal stimulation of 2.5 ± 0.2 -fold ($P < 0.001$). In contrast, in the presence of TGF β_1 , a maximal increase in $\alpha 1(I)$ collagen mRNA levels of 1.5 ± 0.1 -fold ($P < 0.01$) was observed with 5 ng/ml of TGF β_1 , which was not augmented with a higher dose (10 ng/ml). In untreated cells an increase in the basal mRNA levels was observed from 24 to 48 h of incubation, indicating that in the absence of any exogenous treatment, the expression of $\alpha 1(I)$ collagen mRNA increased with time in culture.

Effect of cycloheximide on TGF β_1 - and BMP-2-induced increases in $\alpha 1(I)$ collagen mRNA levels

The effects of TGF β_1 and BMP-2 on $\alpha 1(I)$ collagen mRNA expression were not detectable before 24 h, suggesting that the two factors might not act directly on $\alpha 1(I)$ collagen gene promoter activity. To clarify this point we determined whether new protein synthesis was required in these mechanisms (Figure 2A). After 24 h, in the presence of cycloheximide (0.5 μ g/ml) neither TGF β_1 (5 ng/ml) nor BMP-2 (50 ng/ml) was able to increase $\alpha 1(I)$ collagen mRNA levels, indicating that protein synthesis *de novo* was critical for these cellular responses.

Effects of TGF β_1 and BMP-2 on the transcription rate of the $\alpha 1(I)$ collagen gene

Up-regulation of mRNA levels could result from an increase in the gene transcription rate and/or in mRNA stability. We first analysed the effect of actinomycin D (a transcription inhibitor) on TGF β_1 - and BMP-2-induced increases in $\alpha 1(I)$ collagen mRNA levels (Figure 2B). Cells were treated for 24 h with TGF β_1 (5 ng/ml) or BMP-2 (50 ng/ml) in the presence of vehicle (ethanol) or actinomycin D (3 ng/ml). Actinomycin D abolished the increase in $\alpha 1(I)$ collagen mRNA levels induced by both TGF β_1 and BMP-2, suggesting that a transcriptional event was part of the mechanism. To verify this hypothesis, a transcription assay was performed *in vitro* with nuclei isolated from cells treated with either TGF β_1 (5 ng/ml) or BMP-2 (50 ng/ml) for 48 h. Results from three independent experiments showed that TGF β_1 and BMP-2 up-regulated the transcription rate of the $\alpha 1(I)$ collagen gene by 2.5 ± 0.7 -fold and 3.9 ± 0.9 -fold respectively. Therefore these results confirmed that $\alpha 1(I)$ collagen mRNA levels were transcriptionally controlled by TGF β_1 and BMP-2. In addition, BMP-2 stimulated the transcription rate of the $\alpha 1(I)$ collagen gene to a greater extent than did TGF β_1 , corroborating the results obtained in Northern blot studies suggesting that BMP-2 had a stronger stimulatory effect than TGF β_1 on $\alpha 1(I)$ collagen gene expression.

Effects of TGF β_1 and BMP-2 on $\alpha 1(I)$ collagen mRNA stability

We next determined whether the two factors were also able to affect the stability of $\alpha 1(I)$ collagen mRNA. Cells stimulated with TGF β_1 and BMP-2 were, after a period of 24 h, incubated for up to 32 h in the presence of vehicle or DRB (12.5 μ g/ml), an inhibitor of RNA polymerase II. As shown clearly in Figure 3, neither TGF β_1 nor BMP-2 altered the turnover of the mRNA.

Figure 3 Effects of TGFβ₁ and BMP-2 on the stability of α1(I) collagen (COL I) mRNA

Cells were treated either with TGFβ₁ (5 ng/ml) (□) or BMP-2 (50 ng/ml) (△) or with no addition (○) for 24 h followed by the addition of DRB (12.5 μg/ml). Total RNA was isolated at the indicated time points and subjected to Northern blot analysis, as described in the Experimental section. Autoradiographic data were quantified by densitometry, normalized against 28 S rRNA values and expressed as a percentage of COL I mRNA present in cells before the addition of DRB. The figure summarizes the results from three independent experiments (means ± S.D.).

Involvement of protein kinase-dependent pathways in TGFβ₁- and BMP-2-induced increases in α1(I) collagen mRNA expression

Because TGFβ₁ and BMP-2 both up-regulated α1(I) collagen mRNA expression in ROS 17/2.8 cells, it was of interest to examine whether these actions were mediated by comparable signal-transducing cascades. Protein kinases are involved in most of the cytokine signalling pathways. We therefore used several protein kinase inhibitors to assess the role of PKC and PTK activities in the up-regulation of α1(I) collagen mRNA expression by TGFβ₁ and BMP-2.

Effect of PKC inhibitors

Three different inhibitors of PKC were used: staurosporine, calphostin C and chelerythrine chloride. Each of these act on different domains of the PKC molecule (ATP-binding site [21], regulatory domain [22] and catalytic domain [23] respectively) to inhibit the enzyme activity. Although staurosporine is a potent inhibitor of PKC, it also inhibits other kinases, including some tyrosine kinases [21]. In contrast, calphostin C and chelerythrine chloride are two highly specific inhibitors of PKC [22,23].

The cells were treated with TGFβ₁ (5 ng/ml) or BMP-2 (50 ng/ml) in the presence of the different PKC inhibitors or vehicle (DMSO) for the indicated durations. Staurosporine (12.5 and 25 nM) markedly decreased the stimulatory effects of TGFβ₁ and BMP-2 on α1(I) collagen mRNA levels. Thus staurosporine (25 nM) inhibited the response to TGFβ₁ and BMP-2 respectively by approx. 67% and 56% (see Figure 4A). Similarly, chelerythrine chloride (5 μM) inhibited the effects of TGFβ₁ and BMP-2 by approx. 46% and 33% respectively (see Figure 4B), confirming that these cellular responses were mediated by PKC-dependent activities. Agents such as chelerythrine chloride have previously been used in analogous studies under incubation conditions similar to ours to demonstrate the signalling role of PKC in other cellular models [24]. Nevertheless, to confirm the

Figure 4 Effects of PKC inhibitors on the TGFβ₁- and BMP2-induced increases in α1(I) collagen (COL I) mRNA levels

Cells were treated or not (control, C) with TGFβ₁ (T, 5 ng/ml) or BMP2 (B, 50 ng/ml), in the presence of vehicle (DMSO) or (A) staurosporine (12.5 and 25 nM), (B) chelerythrine chloride (5 μM) or (C) calphostin C (400 nM) for 48 h (A, B) or 24 h (C). Northern blot analysis of isolated total RNA was performed, as described in the Experimental section. The autoradiograms shown are representative of those from at least three similar experiments.

involvement of PKC in our system, calphostin C (400 nM) was employed and was used in shorter incubations. This PKC inhibitor also strongly decreased the effects of TGFβ₁ and BMP-2 on α1(I) collagen gene expression, by approx. 86% and 60% respectively (see Figure 4C).

Effect of tyrosine kinase inhibition

We also tested the participation of PTK in these signalling pathways (Figure 5). The cells were treated as described above with increasing doses of genistein (a tyrosine kinase inhibitor) or vehicle. Genistein has previously been used in incubation conditions similar to ours to implicate PTK actions in other cell systems [24]. In the presence of genistein (15 μg/ml), TGFβ₁ and BMP-2 effects on α1(I) collagen mRNA expression were blocked,

Figure 5 Effect of genistein, a PTK inhibitor, on the TGF β_1 - and BMP2-induced increases in $\alpha 1(I)$ collagen (COL I) mRNA levels

Cells were treated or not with TGF β_1 (5 ng/ml) or BMP2 (50 ng/ml) for 48 h, in the presence of increasing doses of genistein. Northern blot analysis of isolated total RNA was performed, as described in the Experimental section. A representative autoradiogram from three independent experiments is shown.

Figure 6 Effects of TGF β_1 and BMP-2 on MAPK (ERK) activity in the absence or presence of genistein

Cultures were preincubated for 3 h in the presence of vehicle alone (DMSO) or vehicle plus genistein (30 μ g/ml). Cells were then treated with serum alone (control, C), TGF β_1 (T, 5 ng/ml) or BMP-2 (B, 50 ng/ml) for 30 min. Cell lysates were immunoprecipitated with anti-ERK antibodies and immunoprecipitates were subjected to kinase assay *in vitro* with MBP as substrate. Phosphorylated MBP was resolved by SDS/PAGE and detected by autoradiography. The autoradiograms shown are representative of two similar experiments.

indicating that genistein-sensitive PTK activities were involved in these transducing cascades.

No change in ROS 17/2.8 cell viability was observed after treatment with staurosporine, calphostin C, chelerythrine chloride or genistein (see the Experimental section).

Effects of TGF β_1 and BMP-2 on MAPK (ERK) activity

PKC and PTK have been shown to participate in the protein phosphorylation cascades leading to the activation of MAPK family members [25]. In addition, the stimulation of ERK has been involved in TGF β_1 -induced gene expression in other cell types. Consequently, we assessed whether TGF β_1 - and BMP-2-induced increases in $\alpha 1(I)$ collagen gene expression in ROS 17/2.8 cells could be correlated with a modification of MAPK (ERK) activity.

With the use of the same culture conditions as in previous experiments, cells were treated with TGF β_1 (5 ng/ml) or BMP-2 (50 ng/ml) for 30 min (Figure 6) in the presence of either genistein (30 μ g/ml) or vehicle (DMSO). ERK activity was determined by immune complex kinase assay with MBP as a substrate. An increase in ERK activity was observed at 30 min in response to TGF β_1 and BMP-2 (approx. 2-fold and 3.5-fold respectively) compared with the control (serum alone). These effects on ERK activity were decreased by approx. 60–70% in

Figure 7 Effects of MEK-1^{Ala-97} expression on TGF β_1 - and BMP-2-induced increases in $\alpha 1(I)$ collagen mRNA levels

(A) Cells were treated with serum alone (control, C) or with TGF β_1 (T, 5 ng/ml) or BMP-2 (B, 50 ng/ml) for 30 min. Cell lysates were immunoprecipitated with anti-ERK antibodies and immunoprecipitates were subjected to kinase assay *in vitro* with MBP as substrate. Phosphorylated MBP was resolved by SDS/PAGE and detected by autoradiography. (B) Cells were treated or not (control, C) with TGF β_1 (T, 5 ng/ml) or BMP-2 (B, 50 ng/ml) for 48 h. Northern blot analysis of isolated total RNA was performed, as described in the Experimental section. The autoradiogram in each panel is representative of two similar experiments.

the presence of genistein. Genistein also had a slight inhibitory effect on the response in the presence of the serum alone. These observations indicated that TGF β_1 and BMP-2 similarly modulated ERK activity under these experimental conditions. Therefore the up-regulation of $\alpha 1(I)$ collagen gene expression by TGF β_1 and BMP-2 was correlated with an early stimulatory effect on ERK activity; genistein inhibited both of these effects.

When the same experiment was repeated with the PKC inhibitor chelerythrine chloride (10 μ M) in place of genistein, chelerythrine chloride did not notably affect the TGF β_1 - or BMP-2-induced increase of ERK activity, in contrast with the effect of genistein (results not shown).

Effects of MEK-1^{Ala-97} expression on the regulation of the $\alpha 1(I)$ collagen gene by TGF β_1 and BMP-2

To examine further the role of the MAPK pathway in the regulation of $\alpha 1(I)$ collagen gene expression by TGF β_1 and BMP-2, the dominant-negative MEK-1 (MEK-1^{Ala-97})-expressing cell lines were established (see the Experimental section). As shown in Figure 7(A), the stimulation of ERK activity by serum alone (control), TGF β_1 (5 ng/ml) or BMP-2 (50 ng/ml) was markedly inhibited in cells expressing the mutant MEK-1^{Ala-97} (pCMEK-1^{Ala-97}-1) in comparison with cells transfected with the empty vector (pC-1) only. In parallel, TGF β_1 and BMP-2 were no longer capable of increasing $\alpha 1(I)$ collagen mRNA levels above control in pCMEK-1^{Ala-97}-1 cells (Figure 7B). The same results were observed with the two other cell lines pCMEK-1^{Ala-97}-2 and pC-2 (results not shown). These results indicate that the MEK-1/ERK cascade is important in the up-regulation of

the expression of the $\alpha 1(I)$ collagen gene by TGF β_1 and BMP-2 in these cells.

DISCUSSION

Osteoblast differentiation is characterized by the production of bone organic matrix in which type I collagen is the main component. This extracellular matrix protein is important both for the structural integrity of the matrix and for its calcification (reviewed in [1]). The capacity of TGF β_1 to control the production and the remodelling of extracellular matrix in most tissues is well documented [26]. In ROS 17/2.8 osteoblast-like cells, TGF β_1 has been shown to up-regulate the expression of genes such as those for osteonectin, osteopontin and type I collagen. Here we report that BMP-2, like TGF β_1 , up-regulates the expression of the $\alpha 1(I)$ collagen gene in this osteoblastic cell line. Furthermore, we demonstrate that the actions of TGF β_1 and BMP-2 on the expression of this early marker of osteoblast differentiation are both mediated by transcriptional events. A number of investigations have shown that the effects of TGF β_1 on gene expression can be exerted at the transcriptional level. Thus TGF β_1 increases the promoter activity of plasminogen activator inhibitor 1 (PAI-1), $\alpha 1(I)$ collagen and $\alpha 2(I)$ collagen genes in fibroblasts [27,28]. Our results confirmed the transcriptional action of TGF β_1 on $\alpha 1(I)$ collagen gene expression in osteoblast-like cells and indicated that BMP-2, too, stimulated $\alpha 1(I)$ collagen mRNA expression by regulating the transcription rate of the gene. It seems likely that TGF β_1 and BMP-2 did not exert a direct effect on the transcription of the $\alpha 1(I)$ collagen gene in ROS 17/2.8 cells because their effect was not detectable before 24 h and required new protein synthesis. This is consistent with a requirement for the new synthesis of nuclear transcription factors. One of the important nuclear factors involved in the regulation of $\alpha 1(I)$ collagen expression is the transcription factor activator protein 1 (AP-1) [29]. AP-1 has also been implicated in the effect of TGF β_1 on the expression of several genes such as those for PAI-I [30], collagenase [31], osteocalcin [32] and $\alpha 2(I)$ collagen [33]. Moreover, in osteoblast-like cells, the expression of AP-1 components such as c-Fos can be regulated by either TGF β_1 [34] or BMPs (BMP-2/BMP-3) [35]. Further studies are now under way to determine the role of AP-1 transcription factors in the TGF β_1 - and BMP-2-induced up-regulation of $\alpha 1(I)$ collagen mRNA expression in ROS 17/2.8 osteoblastic cells.

An analysis of the signalling pathways involved in TGF β_1 - and BMP-2-induced increases in $\alpha 1(I)$ collagen mRNA levels showed that these actions were dependent at least in part on PKC activities. This was demonstrated by using three structurally unrelated PKC inhibitors (staurosporine, chelerythrine chloride and calphostin C) incubated under different conditions (for 24 or 48 h). PKC has a central role in several signal-transducing cascades [36] and has been linked to the up-regulation by TGF β of other proteins such as PAI-1 and fibronectin in human lung carcinoma cells (A549) [9]. However, previous studies have reported that treatment with PMA leads to a down-regulation of the $\alpha 1(I)$ collagen gene expression [37] in osteoblastic cells. However, these studies did not confirm whether, under the conditions of incubations used, PKC activity was up-regulated or down-regulated in response to the PMA added. Moreover, it is important to note that PKC consists of a large family of isoenzymes that are selectively activated depending on the agonist and the cell line [38]. Phorbol esters, in contrast, are less selective than natural agonists towards the different PKC isoforms. Additionally, depending on the cell line, PMA has variable effects on isoenzymes belonging to an atypical group such as PKC ζ [39]. It has, for example, been reported that two distinct

PKC isoforms (PKC δ and PKC ϵ), induce opposite cell responses and different susceptibilities to PMA, even in the same cell line [40]. Therefore the PKC isoenzymes activated by the phorbol ester PMA might well be different from those activated by the natural agonist TGF β_1 (or BMP-2) and therefore trigger different biological responses in cells. A detailed analysis of the activation of the specific PKC isoenzymes involved in each case will provide more insight into this apparent discrepancy.

PTK activities also participated in the signalling cascades involved in the up-regulation of $\alpha 1(I)$ collagen mRNA expression by TGF β_1 and BMP-2, as shown by the inhibitory effect of genistein on this cellular response. Thus the modulation of $\alpha 1(I)$ collagen gene expression by TGF β_1 and BMP-2 in ROS 17/2.8 cells was dependent, at least in part, on both PTK and PKC activities, which have been shown to lead to activation of the MAPK pathway [25].

Recent studies have demonstrated the involvement of MAPK family members such as ERK and Jun kinase in the TGF β signalling pathway [11–13]. MAPK family members are known for their role in the activation of targeted transcription factors that induce the expression of early genes such as *c-fos* [36]. Our work revealed that up-regulation of $\alpha 1(I)$ collagen mRNA expression by TGF β_1 and BMP-2 in ROS 17/2.8 osteoblast-like cells was correlated with an early increase in MAPK (ERK) activity. In contrast, when the action of TGF β_1 and BMP-2 on Jun kinase activity was also examined, no significant effect was observed (results not shown). Additionally, the stimulation of ERK activity by TGF β_1 and BMP-2 was markedly decreased in the presence of genistein, indicating that these actions were also at least partly mediated through upstream activation of PTK. These results support the hypothesis that MAPK (ERK) pathways could be involved in the TGF β_1 and BMP-2 signalling leading to the up-regulation of $\alpha 1(I)$ collagen mRNA in ROS 17/2.8 cells. This hypothesis was verified by using transfected ROS 17/2.8 cell lines expressing a dominant-negative MEK-1 (MEK-1^{Ala-97}) in which the effects of TGF β_1 and BMP-2 on $\alpha 1(I)$ collagen mRNA levels were noticeably decreased.

In summary, we have provided evidence demonstrating the participation of a pathway dependent on PKC, PTK and MEK-1/ERK in the up-regulation of $\alpha 1(I)$ collagen gene expression by TGF β_1 and BMP-2 in ROS 17/2.8 cells. Our findings show that in these cells, BMP-2 and TGF β_1 , which signal through discrete cell-surface receptors, are able to trigger analogous, if not identical, transducing cascades leading to comparable actions on osteoblast-specific gene expression. No other transducing elements have been found to participate in the BMP-2 (or BMP-4) signalling pathway downstream of its receptor, except for the MAPK kinase kinase TGF β -activated kinase 1 and the SMAD proteins. This study therefore demonstrates for the first time that PKC and PTK-dependent activities can participate in a BMP-2-stimulated cell response. We also show for the first time that BMP-2, like TGF β_1 , is able to modulate MAPK (ERK) activity and that the MEK-1/ERK pathway participates in the regulation of $\alpha 1(I)$ collagen gene expression by TGF β_1 and BMP-2 in osteoblastic cells. Further identification of the intermediate kinases and regulatory proteins in these signal transduction pathways should lead us to a better understanding of the mechanisms involved in the regulation of gene expression in the osteoblast by TGF β_1 and BMP-2.

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