Mitogenic G_i Protein-MAP Kinase Signaling Cascade in MC3T3-E1 Osteogenic Cells: Activation by C-Terminal Pentapeptide of Osteogenic Growth Peptide [OGP(10–14)] and Attenuation of Activation by cAMP

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Abstract In osteogenic and other cells the mitogen-activated protein (MAP) kinases have a key role in regulating proliferation and differentiated functions. The osteogenic growth peptide (OGP) is a 14 mer mitogen of osteogenic and fibroblastic cells that regulates bone turnover, fracture healing, and hematopoiesis, including the engraftment of bone marrow transplants. It is present in the serum and extracellular fluid either free or complexed to OGP-binding proteins (OGPBPs). The free immunoreactive OGP consists of the full length peptide and its C-terminal pentapeptide OGP(10–14). In the present study, designed to probe the signaling pathways triggered by OGP, we demonstrate in osteogenic MC3T3 E1 cells that mitogenic doses of OGP(10–14), but not OGP, enhance MAP kinase activity in a time-dependent manner. The OGP(10–14)-induced stimulation of both MAP kinase activity and DNA synthesis were abrogated by pertusis toxin, a G_i protein inhibitor. These data offer direct evidence for the occurrence in osteogenic cells of a peptide-activated, mitogenic Gi protein-MAP kinase-signaling cascade. Forskolin and dBu$_2$-cAMP abrogated the OGP(10–14)-stimulated proliferation, but induced only 50% inhibition of the OGP(10–14)-mediated MAP kinase activation, suggesting additional MAP kinase-dependent, OGP(10–14)-regulated, cellular functions. Finally, it is demonstrated that OGP(10–14) is the active form of OGP, apparently generated proteolytically in the extracellular milieu upon dissociation of OGP–OGPBP complexes. J. Cell. Biochem. 81:594–603, 2001. © 2001 Wiley-Liss, Inc.

Key words: forskolin; osteoblast; mitogenic signaling; pertusis toxin; proliferation; growth factor activation

In mammalian cells, the family of mitogen-activated protein (MAP) kinases provides a key link between membrane bound receptors and changes in the pattern of gene expression. The MAP kinases are activated downstream of many different types of receptors, among them are the tyrosine kinase receptors, cytokine receptors, and serpentine G-protein coupled receptors (GPCR) [Gutkind, 1998; Hipskind and Bilbe, 1998]. In osteogenic cells, members of the MAP kinase family mediate the action of factors such as fibroblast growth factor, platelet derived growth factor (PDGF), insulin-like growth factor, interleukin 6, and fluoride [Bellido et al., 1997; Zhen et al., 1997; Chaudhary and Avioli, 1998; Lau and Baylink, 1998; Kawane and Horiuchi, 1999; Zhang et al., 1999]. Subsequent to their phosphorylation, the activated MAP kinases are translocated to the nucleus and target transcription factors

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This article published online in Wiley InterScience, March 15, 2001.

such as Elk-1 and SAP-1a which activate immediate early genes, particularly members of the *fos* and *jun* proto-oncogene family [Hipskind and Bilbe, 1998].

The osteogenic growth peptide,(OGP) initially isolated from the osteogenic phase of post-ablation regenerating bone marrow [Bab et al., 1992], is a 14-amino acid peptide that increases bone mass [Bab et al., 1992; Chen et al., 2000], enhances fracture healing [Sun and Ashhurst, 1998; Brager et al., 2000], and stimulates blood and bone marrow cellularity, including the engraftment of bone marrow transplants [Gurevitch et al., 1996]. It is a post-translational cleavage product of PreOGP that is generated via alternative translational initiation at a downstream initiation codon of histone H4 mRNA [Bab et al., 1999b]. The OGP is present in micromolar concentrations in the serum of mammals including human [Bab et al., 1992; Greenberg et al., 1995] and is secreted by cultured cells such as NIH 3T3 fibroblasts, non-transformed osteogenic MC3T3 E1 cells [Greenberg et al., 1997], and ROS 17/2.8 osteosarcoma cells [Bab et al., 1999b]. In cell culture models, OGP regulates cell proliferation, alkaline phosphatase activity, and matrix mineralization [Bab et al., 1992; Robinson et al., 1995].

In the serum and in tissue culture medium of NIH 3T3 and MC3T3 E1 cells OGP is present free or complexed to OGP binding proteins (OGPBPs) [Bab et al., 1992, 1999a; Greenberg et al., 1995; Gavish et al., 1997; Greenberg et al., 1997]. The free, mitogenically active form occurs as both the full length, 14-mer peptide and the C-terminal pentapeptide, OGP(10−14) (Tyr-Gly-Phe-Gly-Gly) [Bab et al., 1999a]. OGP(10−14) is the minimal OGP-derived sequence that retains the mitogenic activity of OGP and its in vivo effects [Chen et al., 2000], but does not bind to the OGPBPs [Bab et al., 1999a].

OGP(10−14) shares some biological activities with opiate peptides [Karchenko and Bagrov, 1986; Kharchenko et al., 1989]. In addition, a recent structure/function analysis indicates that, as in the case of opiate peptides, the respective phenolic group and aromatic ring of Tyr[10] and Phe[12] are crucial for the OGP(10−14) activity [Yamazaki et al., 1993; Chen et al., 2000]. These similarities prompt us to hypothesize that like several opiate peptides, the intracellular signaling cascade elicited by OGP(10−14) involves a G_i protein-dependent activation of MAP kinases [Burt et al., 1996; Hawes et al., 1998]. OGP(10−14) is the proteolytic cleavage product of OGP [Bab et al., 1999a, 1999b]. We, therefore, also hypothesized that it is the physiologic active form of OGP generated upon dissociation of the OGP−OGPBP complexes. Indeed, we demonstrate that in MC3T3 E1 osteogenic cells OGP(10−14), but not OGP, activates the MAP kinase pathway. Both OGP(10−14)-mediated MAP kinase activation and stimulation of cell replication were inhibited by pertusis toxin (PTX), thus providing direct evidence for the presence in osteogenic cells of a peptide-regulated, G_i protein-MAP kinase mitogenic signaling cascade.

METHODS

Peptide Synthesis

OGP and OGP(10−14) were prepared according to the standard solid phase peptide synthesis methodology on 4-methyl benzhydrylamine (pMBHA) resin [Barany and Merrifield, 1979] in an Applied BioSystems 430A automated peptide synthesizer. Side chain deprotection with concomitant cleavage from the resin by liquid HF in the presence of 5% anisole generated the crude peptide that was purified by reverse-phase HPLC. The purity of the peptides exceeded 97%. Their integrity was confirmed by mass spectrometry and amino acid analysis.

Cell Culture

Osteogenic MC3T3 E1 cell cultures were maintained in alpha minimal essential medium (αMEM) supplemented with 10% fetal calf serum (FCS) at 37°C in CO_2-air. The cells were subcultured twice a week.

Cell Proliferation

Cell proliferation was assessed essentially as described previously [Bab et al., 1992, 1999a; Greenberg et al., 1993, 1997]. Briefly, cells were seeded in 16-mm multiwell dishes at 10^4 cells/cm^2. After 46 h incubation in αMEM supplemented with 10% FCS the cells were kept for 2 h in serum free medium in the presence or absence of 4% bovine serum albumin (BSA) and PTX, washed twice with PBS and further incubated in 4% BSA-containing medium with or without OGP or OGP(10−14) for an additional 48 h period. In experiments testing the

effect of forskolin (FSK) and dibutryl-cAMP (dBu_2-cAMP) on the stimulation of cell proliferation the cells were challenged with these agents for the last 48 h period in the presence or absence of OGP(10–14). Cell counts were carried out using a hemocytometer.

DNA Synthesis

DNA synthesis was measured using 5-bromo-2′-deoxy-uridine (BrdU) Detection kit III (Boeringer Mannheim, IN). For this assay, cells were cultured in 96-well microtiter plates. Otherwise, the culture protocol was the same to that described above for assessing proliferation. BrdU, 10 μM per well, was added 36 h after supplementing the medium with BSA. BrdU incorporation into DNA was determined 2 h later by ELISA according to the manufacturer's instructions.

Cell Extracts

Cells were seeded in 10 cm dishes at 5×10^5 cells per dish and incubated in 10% FCS supplemented αMEM. Subconfluent cultures were serum-starved overnight in 0.1% BSA-containing αMEM. After additional 2 h incubation with PTX or 15 min with FSK or dBu_2-cAMP the quiescent cultures were washed with phosphate buffered saline (PBS) and incubated in the same medium with OGP(10–14) or OGP. The cells were then rinsed with ice cold PBS, lysed with lysis buffer (50 mM Tris-HCl, pH 7.5, 1 mM EGTA, 150 mM NaCl, 50 mM β-glycerophosphate, 1 mM NaF, 1 mM sodium orthovanadate, 0.5 μg/ml leupeptin, 0.5 μg/ml aprotonin, 0.5 mM PMSF and 1% Triton X-100) and scraped off using a rubber policeman. Lysates were clarified by centrifugation at 15,000 × g for 10 min at 4°C and the supernatant was used as the cytosolic extract for the MAP kinase assay.

MAP Kinase Activity

MAP kinase activity was measured with a MAP kinase enzyme assay kit (Cat no. 17-133, Upstate Biotechnology Inc., Lake Plasid, NY) as the rate of phosphorylating myelin basic protein (MBP), a highly selective substrate for members of the MAP kinase family [Shibutani et al., 1997; Wilmer et al., 1997]. Briefly, cell extracts (800 μg at a concentration of 1 mg/ml) were incubated overnight with a 1:1600 dilution of anti-MAP kinase antibody generated against p[42,43]/

p[44]MAP kinase [anti-rat MAP kinase R2 (ERK1-CT), polyclonal IgG, cat no. 06-182, Upstate Biochemistry Inc., Lake Plasid, NY] at 4°C and for additional 2 h with protein A-Sepharose. Protein A bound immune complexes were then collected by centrifugation, washed three times with lysis buffer and twice with ice-cold kinase buffer (20 mM HEPES, 25 mM β-glycerophosphate, 5 mM EGTA, 1 mM sodium orthovanadate and 1 mM DTT, pH 7.4) and resuspended in 40 μl of the same buffer. Aliquots (10 μl) of the immune complex suspension were mixed with 30 μl of substrate buffer (containing HEPES, ATP, $MgCl_2$, and 20 μg MBP) and 1 μCi [γ-^{32}P]ATP for 10 min at 30°C. The reaction was terminated by adding Laemmli SDS-sample buffer and the samples were boiled for 5 min and resolved on 12% SDS-PAGE. Gels were stained with Coomassie Brilliant Blue, dried, and subjected to autoradiography. The intensities of the stained and radiographic MBP bands were measured by a laser densitometer. MAP kinase activity was expressed as the ratio of optical densities of the radiographic and Coomassie stained bands.

RESULTS

To assess the involvement of G_i proteins in the OGP intracellular signaling, the osteogenic MC3T3 E1 cells, which are sensitive mitogenic responders to OGP [Bab et al., 1992; Greenberg et al., 1993, 1997] were treated with PTX prior to their exposure to exogenously added OGP or OGP(10–14). PTX, an established selective inhibitor or G_i proteins [Rens-Domiano and Hamm, 1995], inhibited dose-dependently both the OGP- and OGP(10–14)-triggered increase in cell number and DNA synthesis (EC_{50}=0.5 pM). Complete inhibition of the OGP-induced growth occurred at PTX concentrations of 0.1–10.0 ng/ml (Figs. 1A–C). Progressive increase of OGP concentrations (10^{-13}–10^{-6} M) failed to rescue the cell growth from the PTX-induced inhibition (Fig. 1D), indicating the absence of competitive binding between PTX and OGP. That in the MC3T3 E1 cells PTX discriminates the G_i protein cascade from other signaling pathways is illustrated by the toxin's failure to interfere with the mitogenic activity of PDGF (Fig. 1E) which is mediated by a receptors tyrosine kinase (RTK) [Williams, 1989].

The vast majority of mitogenic signals, in osteogenic and other cells, are coupled to the
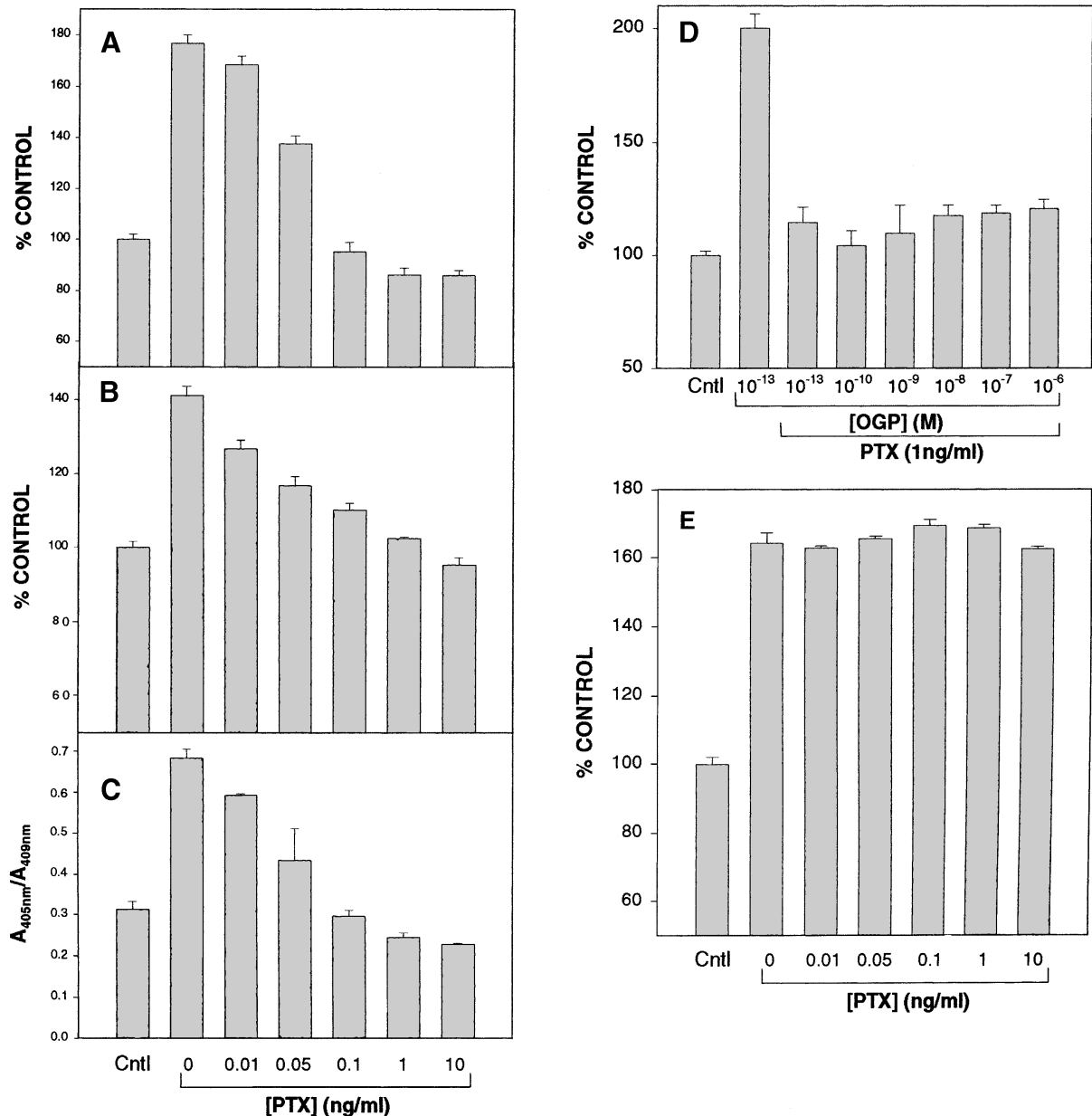
Fig. 1. PTX inhibits OGP- and OGP(10–14)-stimulated osteogenic MC3T3 E1 cell growth and DNA synthesis. Prior to peptide challenge cells were incubated for 2 h with the indicated PTX doses. **(A–C)**: dose-dependent inhibition of 10^{-13} M (optimal peptide dose) OGP (A) or OGP(10–14) (B) induced increase in cell number or of 10^{-13} M OGP stimulation of BrdU uptake (C). **(D)**: increasing OGP doses in the presence of PTX do not stimulate cell growth. **(E)**: PTX at the same dose range does not inhibit 10^{-9} M PDGF stimulated MC3T3 E1 cell growth. Cntl, peptide- and PTX-free control cultures. Data are mean ± SE obtained in three culture wells per condition.

MAP kinase cascade [Gutkind, 1998; Hipskind and Bilbe, 1998]. Therefore, we hypothesized that further downstream signaling by OGP may involve OGP-induced activation of MAP kinases. To test this hypothesis quiescent MC3T3 E1 cells were challenged for 10 min with 10^{-13} M OGP or OGP(10–14). This concentration, of either peptide, has been repeatedly shown to elicit a peak proliferative response in this cell system [Bab et al., 1992, 1999a; Greenberg et al., 1993, 1997]. As shown in Figure 2, only OGP(10–14), not the full
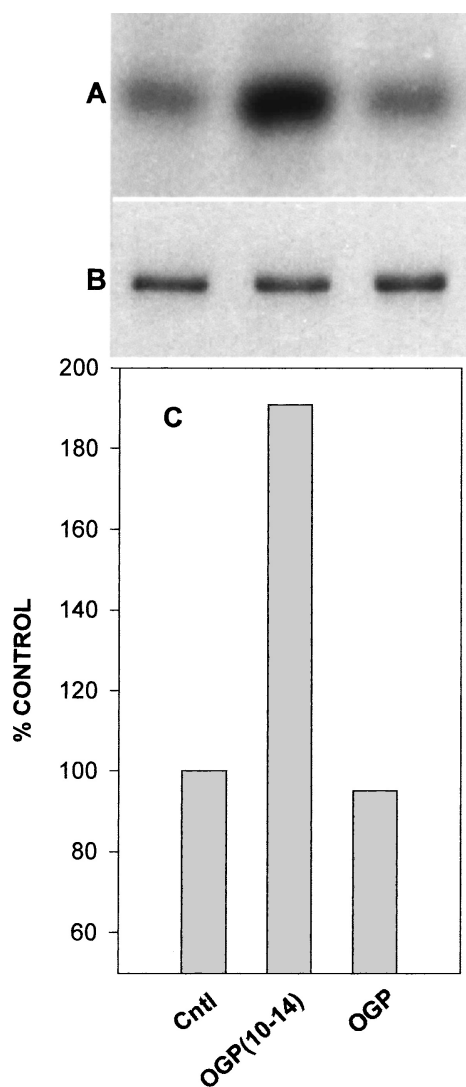
Fig. 2. OGP(10–14), not OGP, activates MAP kinase in osteogenic MC3T3 E1 cells. Quiescent cells were incubated for 10 min with 10^{-13} M OGP or OGP(10–14). Cells were then lysed, and MAP kinases immunoprecipitated and assayed for MBP phosphorylating activity (^{32}P incorporation). **(A)**: autoradiograph showing phosphorylated MBP bands; **(B)**: staining of same bands shown in A; **(C)**: ratio of autoradiography/ Coomassie staining band densities. Cntl, peptide-free cultures. Representative of three repetitive experiments.

length OGP, stimulated MAP kinase activity. OGP(10–14) was thus used in the further experiments which showed 2- to 3-fold MAP kinase activation over the basal level (Figs. 2–4, 6). This effect was dose- and time-dependent with peak stimulation observed at 10^{-13} M peptide concentration (Fig. 3A–C) after 5– 20 min and 2 h (Fig. 3D–F).

To directly test whether the OGP(10–14)-mediated MAP kinase activation is dependent on OGP(10–14)-induced G_i protein signaling, MC3T3 E1 cells were pretreated with PTX prior to challenging with OGP(10–14). The PTX pretreatment completely inhibited the OGP(10–14)-stimulated MAP kinase. The effect of PTX on basal MAP kinase activity was insignificant (Fig. 4).

In different cellular systems DNA synthesis and proliferation as well as MAP kinase activity are either stimulated or inhibited by cAMP [Cook and McCormick, 1993; Siddhanti et al., 1995; Verheijen and Defize, 1997; Cole, 1999, Han and Conn, 1999; Soeder et al., 1999]. We found that incubating the MC3T3 E1 cells with various concentrations of FSK, a stimulator of intracellular cAMP, or the stable synthetic cAMP analog dBu$_2$-cAMP attenuated the OGP(10–14)-stimulated proliferation dose-dependently (Fig. 5). Complete inhibition of the stimulated cell growth occurred at 10^{-4} M dBu$_2$-cAMP and 5×10^{-5} M FSK. At these concentrations, either treatment reduced the OGP(10–14)-induced MAP kinase activation by approximately 50% as compared to 25–30% inhibition of the basal cell growth (Fig. 6).

DISCUSSION

This study establishes the occurrence in osteogenic cells of an extracellular peptide-activated mitogenic cascade consisting of PTX-sensitive G-protein(s) and MAP kinases. The present, biphasic dose-response relationship between OGP(10–14) and MAP kinase activation closely resembles the typical dose-response curve between OGP and proliferation [Bab et al., 1992, 1999a; Greenberg et al., 1995, 1997]. In addition, we demonstrate that the same (i) concentration of OGP(10–14) (10^{-13} M) elicits peak responses of both MAP kinase activity and cell proliferation (Figs. 1 and 3) and (ii) PTX dose (10 ng/ml) abrogates both these responses (Figs. 1 and 4), thus showing a direct correlation between G_i protein-dependent, OGP(10–14)-stimulated MAP kinase activity and mitogenesis. Enzymes of the MAP kinase family have a ubiquitous key role in the regulation of cell growth and differentiation. They are activated by a variety of cell surface receptors, in particular tyrosine kinase and GPCRs [Gutkind, 1998]. Of particular relevance to the present findings is that like OGP(10–14), other
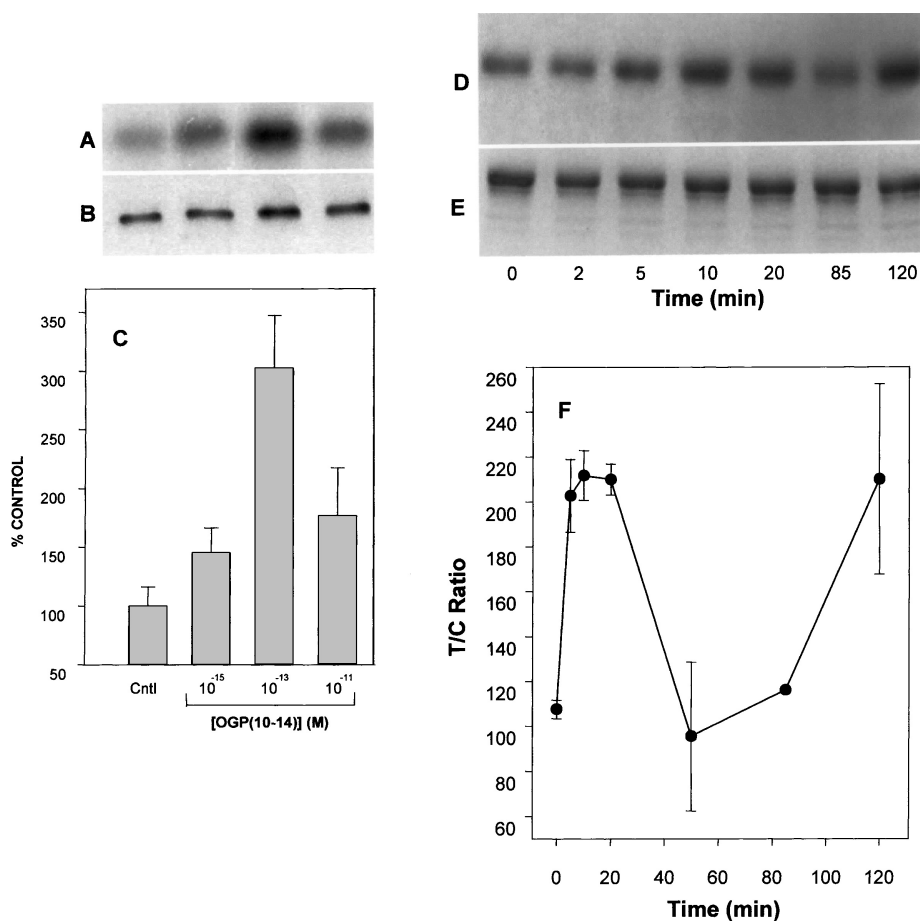
Fig. 3. Dose- and time-dependent OGP(10–14) MAP kinase activation in osteogenic MC3T3 E1 cells. Quiescent cells were incubated for 10 min with the indicated OGP(10–14) concentrations (A–C) or for the indicated time periods with 10^{-13} M OGP(10–14) (D–F). Following incubation the cells were lysed, and MAP kinases immunoprecipitated and assayed for MBP phosphorylating activity (^{32}P incorporation). **(A and D)**: autoradiographs showing phosphorylated MBP bands; **(B and E)**: Coomassie staining of same bands shown in A. **(C and F)**: ratio of autoradiography/Coomassie staining band densities were determined and results obtained in OGP(10–14) treated cultures calculated as percent of peptide free control cultures; data are mean ± SE of four repetitive, independent experiments (80 min time point in F was tested in one experiment only). Cntl, peptide-free control culture.

opiate-like peptides capable of regulating bone regeneration [Rosen and Bar-Shavit, 1994] also activate a G_i protein-MAP kinase signaling cascade [Burt et al., 1996; Hawes et al., 1998, Allouche et al., 1999].

The specific MAP kinases activated by G-proteins, and consequently their downstream targets, depend to a large extent on the particular extracellular agonist and cellular complement of receptors, G-proteins and effectors. For example, while parathyroid hormone (PTH)-dependent activation of MAP kinase has been observed in Chinese hamster ovary (CHO-R15), parietal yolk sac carcinoma (Pys2), and kidney OK cells [Verheijen and Defize, 1997;

Cole, 1999], the hormone inhibited MAP kinase activity in osteosarcoma ROS 17/2.8 and UMR106 cells [Verheijen and Defize, 1995]. Consistent with the present findings, it has been recently suggested that in osteogenic MC3T3 E1 cells G_i protein-activating, mitogenic agonists (i.e., fluoride and epinephrine) regulate extracellular signal-regulated kinase (ERK) members of the MAP kinase family [Caverzasio et al., 1997; Lau and Baylink, 1998; Suzuki et al., 1999]. On the other hand, it has been proposed that alkaline phosphatase activity in these cells is regulated via a G_i protein-dependent p38 MAP kinase [Suzuki et al., 1999].
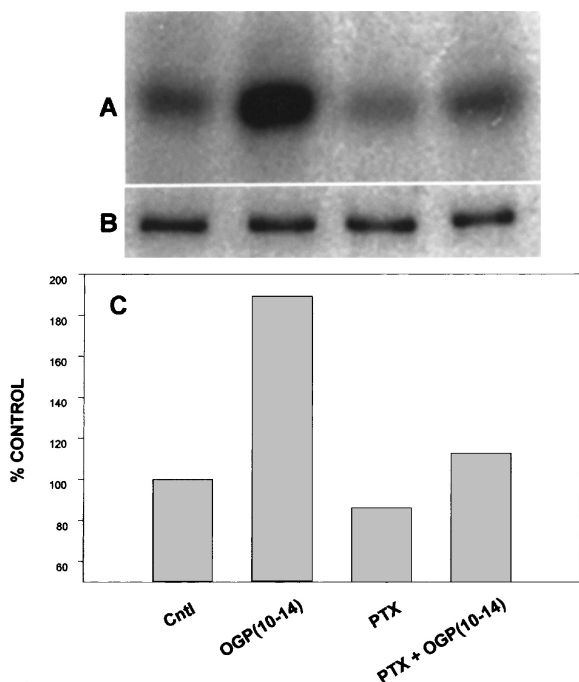
Fig. 4. PTX inhibits OGP(10–14)-mediated MAP kinase activation in osteogenic MC3T3 E1 cells. Quiescent cells were preincubated with 10 ng/ml PTX for 2 h, washed and treated for additional 10 min with 10^{-13} M OGP(10–14). Cells were then lysed, and MAP kinases immunoprecipitated and assayed for MBP phosphorylating activity (^{32}P incorporation). (**A**): autoradiograph showing phosphorylated MBP bands; (**B**): Coomassie staining of same bands shown in A; (**C**): ratio of autoradiography/Coomassie staining band densities. Cntl, peptide- and PTX-free control cultures. Representative of three repetitive experiments.

It has been repeatedly demonstrated that in osteosarcoma and non-transformed osteogenic cells cAMP and cAMP-generating agents such as PTH and FSK attenuate proliferation and MAP kinase activation induced via either G proteins or RTKs [Hakeda et al., 1987; Quarles et al., 1993; Siddhanti et al., 1995; Verheijen and Defize, 1995; Yamaguchi et al., 1988; Chaudhary and Avioli, 1998]. Consistent with these observations our results show that in the osteogenic MC3T3 E1 cells FSK and the stable synthetic cAMP analog dBu$_2$-cAMP inhibit the OGP(10–14)-stimulated proliferation and MAP kinase activation (Figs. 5, 6). The higher inhibition seen in the OGP(10–14)-stimulated cultures (as compared to non-stimulated controls) may be attributed to the role of the autocrine OGP circuit in maintaining basal cell growth [Greenberg et al., 1997]. The significance of these findings relates to the potential points of

convergence of G$_i$ protein- and cAMP-regulated signaling pathways. A substantial body of evidence suggests that mitogenic signals arising from both RTKs and G proteins ubiquitously activate the Ras/MKK/ERK cascade [Marshall, 1995]. In the case of the heterotrimeric, PTX-sensitive G$_i$ proteins it has been further demonstrated that the Gβγ subunits mediate Ras-dependent MAP kinase activation by activating Src family tyrosine kinases and forming a complex with membrane-associated RTKs and cytosolic adapter proteins such as Shc and Grb2 [Crespo et al., 1994; Luttrell et al., 1997; Della Rocca et al., 1999]. Activated by either a G$_i$ protein or RTK, the Ras/MKK/MAP kinase cascade may be modulated by cAMP-regulated mechanisms at several levels downstream of Ras and via a repertoire of cytosolic kinases, thus affecting multiple cell functions [Cook and McCormick, 1993; Hafner et al., 1994; Siddhanti et al., 1995; Han and Conn, 1999; Verheijen and Defize, 1995, 1997; Wu et al., 1993]. Likewise, the present incomplete correlation between the cAMP-induced abrogation of OGP(10–14)-stimulated proliferation and limited inhibition of the OGP(10–14)-induced MAP kinase activation (Figs. 5, 6) suggests that in osteogenic cells functions other than proliferation are also regulated by the OGP(10–14)-MAP kinase pathway. Such functions may include the reported OGP enhancement of alkaline phosphatase and expression of transforming growth factor β [Bab et al., 1992; Robinson et al., 1995; Brager et al., 2000].

Figure 7 depicts a model of OGP proteolytic activation and signaling. Post-translationaly, PreOGP is proteolytically processed to yield the OGP, which in the extracellular milieu forms a complex with OGPBPs [Bab et al., 1992, 1999a, 1999b; Greenberg et al., 1995, 1997; Gavish et al., 1997]. OGP(10–14), the apparent physiologically active form of OGP, is proteolytically generated upon dissociation of the OGP-OGPBP complexes [Bab et al., 1998; Ruchon et al., 2000], interacts with a yet unidentified receptor and activates the G$_i$ protein-MAP kinase cascade. Although we have previously demonstrated that OGP is the OGP(10–14) precursor [Bab et al., 1999a, 1999b], using lengthy (24–48 h) DNA synthesis and proliferation assays in which exogenously added OGP is exposed to proteolytic cleavage, we were unable to differentiate its activity from that of OGP(10–14). For the present assessment of
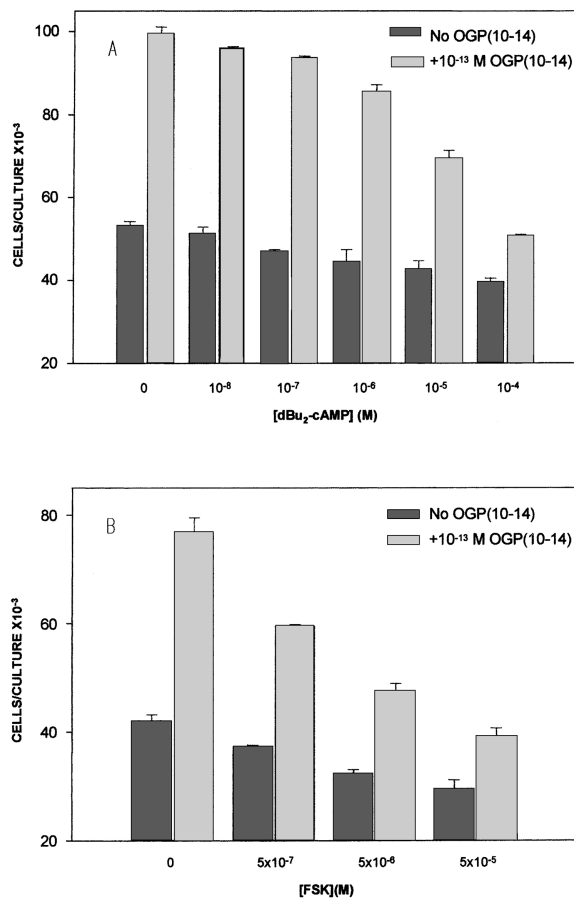
Fig. 5. cAMP inhibits OGP(10–14) stimulated osteogenic MC3T3 E1 cell proliferation. Cells were challenged for 48 h with the indicated doses of dBu$_2$-cAMP **(A)** or FSK **(B)** in the absence or presence of 10^{-13} M OGP(10–14). Data are mean ± SE obtained in three culture wells per condition.

Fig. 6. cAMP inhibits OGP(10–14)-mediated MAP kinase activation in osteogenic MC3T3 E1 cells. Quiescent cells were incubated with 10^{-4} M dBu$_2$-cAMP or 5×10^{-5} M FSK for 15 min prior to 10 min treatment with 10^{-13} M OGP(10–14). Cells were then lysed, and MAP kinases immunoprecipitated and assayed for MBP phosphorylating activity (^{32}P incorporation). **(A)**: autoradiograph showing phosphorylated MBP bands; **(B)**: Coomassie staining of same bands shown in A; **(C)**: ratio of autoradiography/Coomassie staining band densities. Cntl, dBu$_2$-cAMP-, FSK- and peptide-free control culture.

MAP kinase activation the peptides were exposed only briefly (10 min) to the cell culture milieu, evidently shorter than the time necessary for the accumulation of sufficient extracellular peptidase activity to cleave the OGP. We could thus demonstrate that it is the OGP(10–14), not the precursor OGP, that conveys the OGP-like activity to the osteogenic cells (Fig. 2).

In conclusion, the present results provide direct evidence for the occurrence of a peptide-activated, Gi protein-MAP kinase mitogenic signaling cascade in osteogenic cells. In view of the recent demonstration that MAP kinases have a key role in directing mesenchymal stem cells to the osteogenic lineage [Jaiswal et al., 2000], further understanding of the presentl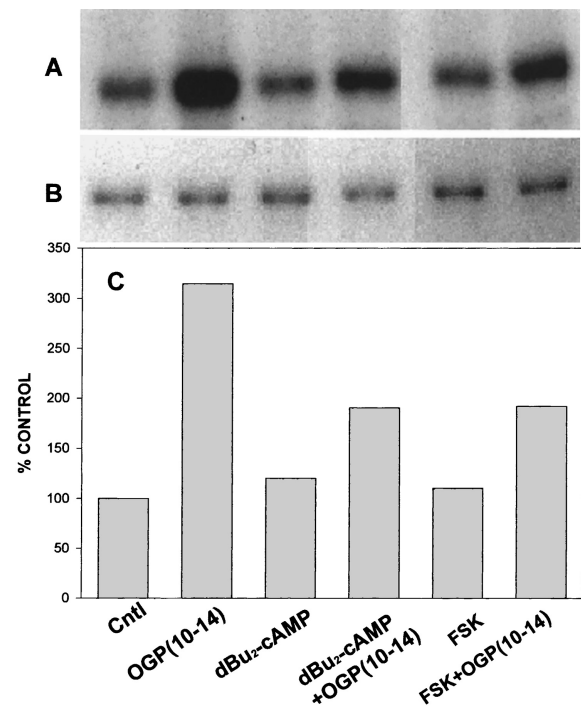y reported and other osteogenic cell MAP kinase related signaling pathways may ultimately provide strategies for selective regulation of proliferation and differentiated functions in these cells.
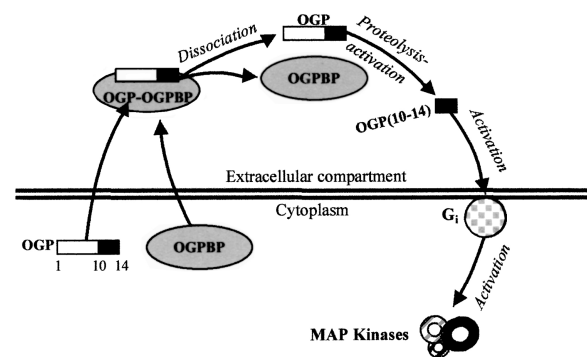

Fig. 7. Model of OGP activation of G$_i$ protein-MAP kinase signaling cascade in osteogenic MC3T3 E1 cells following generation of OGP(10–14) from OGP (upon dissociation of OGP-OGPBP complexes) and its interaction with a putative OGP receptor.

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