ERK1/2-activated de Novo Mapkapk2 Synthesis Is Essential for Osteogenic Growth Peptide Mitogenic Signaling in Osteoblastic Cells*

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In osteoblasts, the mitogen-activated protein kinases ERK1/2 and p38 as well as the cAMP-response element-binding protein (CREB) have been implicated in the regulation of proliferation and differentiation. The osteogenic growth peptide (OGP) is a 14-mer bone cell mitogen that increases bone formation and trabecular bone density and stimulates fracture healing. OGP-(10–14) is the physiologically active form of OGP. Using gene array analysis, real-time reverse transcription-PCR, and immunoblot and DNA synthesis assays we show here that in MC3T3 E1 and newborn mouse calvarial osteoblastic cultures the OGP-(10–14) mitogenic signaling is critically dependent on de novo synthesis of mitogen-activated protein kinase-activated protein kinase-2 (Mapkapk2) mRNA and protein. The increase in Mapkapk2 occurs following short term (5–60 min) stimulation of ERK1/2 activity by OGP-(10–14); phosphorylation of p38 remains unaffected. Downstream of Mapkapk2, CREB is phosphorylated on Ser133 leading to its enhanced transcriptional activity. That these events are critical for the OGP-(10–14) mitogenic signaling is demonstrated by blocking the effects of OGP-(10–14) on the ERK1/2 pathway, Mapkapk2, CREB, and DNA synthesis using the MEK inhibitor PD098059. The OGP-(10–14) stimulation of CREB transcriptional activity and DNA synthesis is also blocked by Mapkapk2 siRNA. These data define a novel mitogenic signaling pathway in osteoblasts whereby ERK1/2 stimulation of CREB phosphorylation and transcriptional activity as well as DNA synthesis are critically dependent on de novo Mapkapk2 synthesis.

In mammalian cells, the family of mitogen-activated protein (MAP)3 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, including tyrosine kinase receptors, cytokine receptors, and serpine G-protein coupled receptors (1, 2). The MAP kinases consist of four subfamilies: the extracellular signal-regulated kinases 1 and 2 (ERK1/2), c-Jun N-terminal kinase/stress-activated kinase, p38 MAP kinase, and ERK 5. Further downstream, they regulate a multitude of transcription factors that control cell proliferation, survival, and differentiation (3, 4). In osteoblasts, ERK1/2-dependent phosphorylation cascades have been implicated in the regulation of proliferation and RUNX2 activity (5, 6). Activation of p38 has been demonstrated in osteoblasts undergoing differentiation after stimulation with bone morphogenetic protein-2 and epidermal growth factor (7, 8).

The osteogenic growth peptide (OGP) is a 14-mer bone cell mitogen that increases bone formation and trabecular bone density and stimulates fracture healing when administered to mice and rats (9–11). Transgenic mice overexpressing OGP have a markedly increased peak bone mass (12). OGP is present in mammalian serum in micromolar concentrations mainly complexed to α2-macroglobulin (13). Upon its dissociation from the complex, it is proteolytically activated yielding the mitogenic C-terminal pentapeptide OGP-(10–14) (14). In addition to stimulating bone formation, OGP-(10–14) potently enhances hematopoiesis (15, 16). We have reported recently (17) that the mitogenic action of OGP-(10–14) involves the activation of a G-protein-MAP kinase signaling pathway.

The present work was undertaken to identify osteoblastic MAP kinases involved in the OGP-(10–14) mitogenic action and to elucidate the OGP-(10–14) signaling pathway downstream of MAP kinase. We have identified a novel OGP-(10–14)-stimulated mitogenic pathway whereby short term activation of ERK1/2 enhances the expression of mitogen-activated protein kinase-activated protein kinase-2 (Mapkap2), resulting in increased phosphorylation and the transcriptional activity of the cAMP response element-binding protein (CREB).

EXPERIMENTAL PROCEDURES

Materials—OGP-(10–14), amino acid sequence Tyr-Gly-Phe-Gly-Gly, molecular mass 499.7 kDa, was supplied by Polypeptides Laboratories, Inc. (Torrance, CA) and provided by Abiogen Pharma Spa (Pisa, Italy). Tissue culture ingredients were from Biological Industries (Beit Haemek, Israel). Fatty acid-free bovine serum albumin was purchased from Sigma (catalog no. A-7030). Collagenase P was purchased from Roche Applied Science. Antibodies to phosphorylated and non-phosphorylated ERK1/2, p38 MAP kinase, Mapkapk2, and CREB were from Cell Signaling Technology (Beverly, MA). The respective PD098059 and SB203580 inhibitors of the ERK1/2-activating kinase MEK and p38 MAP kinase, Mapkapk2, and CREB were from R&D Systems (Minneapolis, MN). 32P]dCTP (10 μCi/μl; 3,000 Ci/mmol) was obtained from Amersham Biosciences. pCEP4 was from Invitrogen. Reagents for luciferase assays were obtained from Promega (Madison, WI). Macroarrays were purchased from Superarray Bio-
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Science Corp. (Bethesda, MD) and siRNAs from Santa Cruz Biotechnology (Santa Cruz, CA). Colorimetric 5-bromo-2′-deoxyuridine (BrdUrd) detection kit III and reagents for real-time RT-PCR were from Roche Diagnostics.

Cell Culture, Western Blotting, RNA Interference, and DNA Synthesis Measurement—Two culture models were employed in this study. MC3T3 E1 osteoblastic cells were maintained as reported previously (17). Newborn mouse calvarial osteoblasts (NeMCO) were prepared from 5-day-old mice by successive collagenase digestion (18). For immunoblot analysis, MC3T3 E1 cells were seeded in 10-cm dishes at 5 × 10⁵ cells/dish and incubated in αMEM supplemented with 10% fetal calf serum. Subconfluent cultures were serum-starved overnight in 0.1% bovine serum albumin containing αMEM. Thereafter, the cells were incubated for various time periods, ranging from 5 min to 8 h, in the same medium with or without OGP-(10–14) and MAP kinase inhibitors. The cells were then rinsed with ice-cold phosphate-buffered saline and lysed with 50 mM Tris-HCl buffer, pH 7.5, 1% Triton X-100, 150 mM NaCl, 1 mM EGTA, 50 mM β-glycerophosphate, 1 mM NaF, 10 μg/ml leupeptin, 10 μg/ml aprotinin, 0.5 mM phenylmethylsulfonyl fluoride, and 1 mM sodium orthovanadate. The cells were then scraped off using a rubber policeman, and the lysates were clarified by centrifugation at 12,000 × g for 15 min. Samples from each lysate, containing 40–120 μg of protein, were fractionated by SDS-PAGE and then electroblotted onto nitrocellulose membranes. The membranes were blocked with nonfat dry milk in TBS/Tween-20. The Western blots were probed with antibodies to phosphorylated ERK1/2, ERK1/2, phosphorylated p38, p38, phosphorylated Mapkapk2, Mapkapk2, phosphorylated CREB, and CREB. Proteins on the Western blots were detected using the LumiGLO chemiluminescent detection system (Cell Signaling).

DNA synthesis was measured as reported previously (17). After serum starvation the cells were incubated in 4% bovine serum albumin of protein, were fractionated by SDS-PAGE and then electroblotted onto nitrocellulose membranes. The membranes were blocked with nonfat dry milk in TBS/Tween-20. The Western blots were probed with antibodies to phosphorylated ERK1/2, ERK1/2, phosphorylated p38, p38, phosphorylated Mapkapk2, Mapkapk2, phosphorylated CREB, and CREB. Proteins on the Western blots were detected using the LumiGLO chemiluminescent detection system (Cell Signaling).

DNA synthesis was measured as reported previously (17). After serum starvation the cells were incubated in 4% bovine serum albumin with or without OGP-(10–14) and with or without inhibitors of MAP kinase phosphorylation. After labeling with BrdUrd for 24 h, its incorporation into DNA was determined using a commercial kit, according to the manufacturer’s instructions.

Transfection and Luciferase Assay—A luciferase construct reporting on CREB transcriptional activity, containing three copies of a canonical CRE (pCRE3-luc) was stably transfected into MC3T3 E1 cells. Resistance to hygromycin B (Calbiochem) was conferred by pCEP4 (Invitrogen) encoding hygromycin B phosphotransferase. Cells were plated in 6-well plates and cotransfected, using the calcium phosphate coprecipitation method, with pCRE3-luc and pCEP4 at a molar ratio of 15:1.

FIGURE 1. OGP-(10–14)-induced stimulation of osteoblastic cell proliferation is mediated by ERK1/2. A–D, MC3T3 E1 cultures. E–F, NeMCO cultures. A, cells were incubated with the indicated OGP-(10–14) concentrations for 5 min, and Western blot analysis was performed with antibodies against phosphorylated ERK1/2 (p-Erk 1/2) (upper panel) or ERK1/2 (lower panel). B, cells were incubated with OGP-(10–14) or vehicle for the indicated time periods, and Western analysis of phosphorylated ERK1/2 (top two panels) and ERK1/2 (bottom panel) was carried out as in A. C, cells were incubated with or without OGP-(10–14) and 75 μM of the MEK inhibitor PD098059 for 15 min, and Western analysis was carried out as in A. D and F, cells were incubated for 48 h with or without OGP-(10–14) and the indicated doses of PD098059 and then analyzed for BrdUrd incorporation into DNA; data are mean ± S.E. obtained in six culture wells per condition. *, p < 0.05 versus cultures treated only with OGP-(10–14). E, cells were incubated with either vehicle (Cntl) or the indicated OGP-(10–14) concentrations. Data are mean ± S.E. obtained in six culture wells per condition. *, p < 0.05 versus control.
Resistant cells were selected by treatment with 200 μg/ml hygromycin B for 20 days. Wells with 1–3 resistant colonies were propagated, and one cell line, in which luciferase activity was stimulated severalfold by forskolin, was chosen for further experiments. To test the effect of OGP-(10–14) on CREB transcripational activity, the stably transfected cells, heretofore MC3T3 E1/CRE-luc, were plated in 48-well plates and grown for 48 h in αMEM supplemented with 10% fetal calf serum. After 2 h of starvation the cells were fed with OGP-(10–14) with or without PD98059 in αMEM containing 4% bovine serum albumin. The cells were harvested 13 h thereafter and lysed in "reporter lysis buffer" (Promega). Luciferase activity was determined using a microtiter plate luminometer (LB940 multilabel reader, Berthold Technologies, Bad Wildbad, Germany).

RNA Interference—Inhibition of Mapkapk2 expression was achieved using a commercial siRNA kit (Santa Cruz Biotechnology) according to the manufacturer’s instructions. The kit included mouse Mapkapk2 siRNA (catalog no. SC-35856), control siRNA (catalog no. SC-37007), siRNA dilution buffer (catalog no. SC-29527), siRNA transfection reagent (catalog no. SC-29528), and siRNA transfection medium (catalog no. SC-36868). Briefly, MC3T3 E1 or NeMCO cells were seeded in 96-well plates, 5 × 10^3 cells/well in 200 μl of antibiotic-free medium. Cultures at ~50% confluence were transfected with control or Mapkapk2 siRNA in transfection medium containing transfection reagent. After 5 and 30 h of incubation in control and Mapkapk2 siRNA, respectively, the cells were serum-starved for 2 h and then challenged with OGP-(10–14).

RNA Isolation—Total RNA was isolated from MC3T3 E1 and NeMCO cells incubated for 4 or 8 h with or without OGP-(10–14) and MAP kinase inhibitors using TRI reagent kit (Molecular Research Center, Inc., Cincinnati, OH) followed by a phenol-chloroform phase extraction and isoproply precipitation. RNA quality was assessed by light absorbance at 260 and 280 nm and by agarose gel electrophoresis and ethidium bromide staining. A similar RNA isolation protocol was applied to cells transfected with siRNA.

cDNA Macroarray Analysis—RNA samples displaying an A_{260/280} ratio of 1.8 or higher and well defined 18 S and 28 S ethidium bromide-stained bands were subjected to expression analysis using mouse MAP kinase signaling pathways gene array kit (GEArray™ Q series, catalog no. MM-017; Superarray Bioscience Corp.). Synthesis of β²P-labeled cDNA and hybridization were carried out according to the manufacturer’s instructions. Digital images were acquired from autoradiographs with a phosphorimaging device (BAS2000, Fuji, Tokyo, Japan) at 200 pixel/inch and formatted (squared, cropped, inverted). The data were extracted using ScanAlayze 2.5 software (Stanford University, Palo Alto, CA). For each macroarray the formatted image was loaded into channel 1 and channel 2 (gain = 1). A grid of spots (10-pixel diameter) was overlaid and adjusted to the array image, and the average pixel intensity (API) within the spot was measured. GEArray Analyzer version 1.2 software (Superarray Bioscience Corp.) was used to calculate gene expression levels for each feature. The usable range of API values was 7,000–65,000. This range was established from the minimum (background) to the maximum (or plateau) API value detected on the radiograph. Gene-specific PCR primers for Mapkapk2 were as follows: sense, 5'–GCCATTTTGAAACTCACTG-3'; antisense, 5'–AGGGACCTTCGTAGATTG-3'; and for β-actin: sense, 5'–GAGACCTTCAACCCCCAGCC-3'; and antisense, 5'–GCCATCTCTTGTCTGAAAGTCT-3'. Expression level of the Mapkapk2 gene was evaluated as the ratio of its mRNA to that of β-actin mRNA.

Statistical Analysis—Analysis of variance was employed for statistical analysis. When significant differences were indicated by analysis of variance, group means were compared using the Student-Newman-Keuls test for pairwise comparisons. The Mann-Whitney U test was used for the cDNA macroarray analysis.

RESULTS
OGP-(10–14) Mitogenic Signaling in Osteoblasts Involves Activation of ERK1/2 but Not p38—Previously, we showed by immunoprecipitation and kinase assays that treatment of osteoblastic cells with OGP-
(10–14) results in stimulation of MAP kinase (17). Because the antibodies used in that study did not differentiate between MAP kinase subtypes, we performed Western analyses to identify the specific MAP kinase involved in the OGP-(10–14) mitogenic signaling. We initially investigated the activation of ERK1/2. Cells challenged for 5 min with different OGP-(10–14) concentrations clearly demonstrated a dose-dependent stimulation of ERK1/2 phosphorylation (Fig. 1A). The strongest signal was observed at 10−13 M, which is also the concentration at which OGP-(10–14) exhibits the strongest effects on both MAP kinase activity and MC3T3 E1 cell proliferation (17, 19). Stimulation of ERK1/2 phosphorylation by OGP-(10–14) at 10−13 M was evident already after 5 min, was strongest after 15 min, and was sustained for at least 60 min (Fig. 1B). As expected, the OGP-(10–14)-induced ERK1/2 phosphorylation was not observed in the presence of PD098059, a specific inhibitor of MEK, the ERK1/2-activating kinase (20) (Fig. 1C).

To investigate whether ERK1/2 phosphorylation is required for the mitogenic activity of OGP-(10–14), we tested the effect of PD098059 on OGP-(10–14)-induced DNA synthesis in the MC3T3 E1 and NeMCO cultures (Figs. 1, D–F). Because OGP-(10–14) had not been tested in the NeMCO model, we initially carried out a dose-response analysis, testing the mitogenic effect of OGP-(10–14) in this system. OGP-(10–14) stimulated BrdUrd incorporation into newly synthesized DNA in these cells with a peak effect at 10−12 M (Fig. 1E). Therefore, the inhibition experiments that followed were carried out at this concentration. As shown in Fig. 1, D and F, the OGP-(10–14) stimulation of BrdUrd incorporation into newly synthesized DNA in the MC3T3 E1 and

### Table One

| Gene    | GenBank™ accession no. | Expression level | -Fold change |
|---------|------------------------|-----------------|--------------|
|         |                        | 4 h | 8 h | 4 h | 8 h |
| Mapkapk2 | X76850                | ++ | ++ | 3.03 ± 1.49 | 5.21 ± 0.10^a |
| Mef2c   | NM_025282             | ++ | ++ | 2.34 ± 0.57 | 2.92 ± 0.40^b |
| NFAT3   | NM_023699             | ++ | ++ | 1.17 ± 0.08 | 2.45 ± 0.80^c |
| Coll1   | U08020                | ++ | ++ | 2.11 ± 0.20 | 1.61 ± 0.03^b |
| Knn1    | NM_032397             | ++ | ++ | 2.21 ± 0.30 | 5.70 ± 0.77^a |

* Symbols indicate relative expression level in control (OGP-(10–14)-free) cultures: ++, high expression; +, medium expression.

^a p < 0.05 for OGP-(10–14)-treated versus control cells.

**FIGURE 3.** Inhibition of ERK1/2 mitigates OGP-(10–14)-induced stimulation of Mapkapk2 expression. MC3T3 E1 (A) and NeMCO (B) cells were incubated with OGP-(10–14) and 75 μM PD098059 for either 4 or 8 h, and Mapkapk2 mRNA levels were analyzed by real-time RT-PCR. Data are mean ± S.E. of 4–6 measurements per condition. *p < 0.05 versus unmarked conditions. Bottom panels are electrophoregrams of ethidium bromide-stained agarose gels showing amplicons retrieved from the real-time RT-PCR reaction. C, MC3T3 E1 cells were incubated with OGP-(10–14) and PD098059 for 8 h followed by Western blot analysis with antibodies against Mapkapk2, phosphorylated Thr222-labeled Mapkapk2, or phosphorylated Thr334-labeled Mapkapk2. NS, nonspecific band of 57 kDa serving as loading control.
NeMCO cells was dose-dependently suppressed by the MEK-ERK1/2 pathway inhibitor PD098059. These results suggest that stimulation of the ERK1/2 pathway is required for the OGP-(10–14) mitogenic signaling.

To rule out the occurrence of an alternative MAP kinase signaling pathway in short-term use of OGP-(10–14), we analyzed p38 activation employing a similar approach. OGP-(10–14) did not stimulate p38 phosphorylation in MC3T3 E1 osteoblastic cells (Fig. 2A). In addition, SB203580, a specific inhibitor of p38 (21), did not inhibit the OGP-(10–14) stimulation of DNA synthesis in these cells even at 75 μM (Fig. 2B).

Identification of OGP-(10–14)-regulated Genes Involved in MAP Kinase Signaling Pathway—To identify signaling pathways downstream of ERK1/2 that are affected by OGP-(10–14), we analyzed p38 activation employing a similar approach. OGP-(10–14) did not stimulate p38 phosphorylation in MC3T3 E1 osteoblastic cells (Fig. 2A). In addition, SB203580, a specific inhibitor of p38 (21), did not inhibit the OGP-(10–14) stimulation of DNA synthesis in these cells even at 75 μM (Fig. 2B).

Identification of OGP-(10–14)-regulated Genes Involved in MAP Kinase Signaling Pathway—To identify signaling pathways downstream of ERK1/2 that are affected by OGP-(10–14), we surveyed changes in gene expression in the MC3T3 E1 cells using a commercially available gene array system designed to analyze the mRNA levels of genes involved in the MAP kinase pathway. OGP-(10–14) significantly up-regulated Mapkapk2, NFAT3, and Mef2c, which encode signaling molecules downstream of the MAP kinase (22–24). In addition, our MAP kinase-related gene array analysis suggests that OGP-(10–14) has a low though significant effect on the expression of Col1α1, which encodes the α1(I) chain of the predominant bone matrix protein type I collagen (25). The expression of Kcnn1, a small-conductance calcium-activated potassium channel (26), was also stimulated by OGP-(10–14) (TABLE ONE).

Regulation of Mapkapk2 Gene Expression by OGP-(10–14)—Of the signaling molecules downstream of the MAP kinase, OGP-(10–14) most strongly stimulated Mapkapk2 mRNA levels, by 3.03- and 5.21-fold following 4 and 8 h, respectively (TABLE ONE). To validate this response, we performed real-time RT-PCR and Western blot analyses of Mapkapk2 in OGP-(10–14)-treated cells. As shown in Fig. 3, A and C, in both the MC3T3 E1 and NeMCO cells Mapkapk2 mRNA was increased by more than 2-fold after 4 and 8 h of treatment, respectively. The up-regulation of Mapkapk2 mRNA resulted in a parallel increase at the protein level (Fig. 3C). We also investigated whether the increase in the Mapkapk2 protein was associated with an alteration in its phosphorylation status. Western analyses with antibodies against phosphorylated Thr222-labeled Mapkapk2 and phosphorylated Thr334-labeled Mapkapk2 yielded essentially the same results as those obtained with the pan antibody, suggesting that the OGP-(10–14)-induced increase in the 47-kDa Mapkapk2 was not associated with alterations in its phosphorylation status (Fig. 3C). The increase in Mapkapk2 mRNA and protein levels were blocked by PD098059 (Fig. 3), indicating that activation of the MEK-ERK1/2 pathway is critical for mediating the OGP-(10–14)-induced stimulation of Mapkapk2 expression.

Mapkapk2 Mediates the Mitogenic Effect of OGP-(10–14)—We used RNA interference to silence the Mapkapk2 gene and determine whether Mapkapk2 plays a role in the OGP-(10–14) mitogenic activity. As in the case of some other genes, such as lamin A/C in HeLa SS6 cells.
(27) and S100A4 in periodontal ligament cells (28), the Mapkapk2 siRNA knocked down the basal Mapkapk2 expression almost completely. It also repressed the OGP-(10–14) stimulation of Mapkapk2 mRNA (Fig. 4A). Likewise, compared with control siRNA, Mapkapk2 siRNA mitigated the stimulatory effect of OGP-(10–14) on DNA synthesis in both the MC3T3 E1 and NeMCO culture systems (Fig. 4B and C), indicating that Mapkapk2 is necessary for the mitogenic effect of OGP-(10–14). Interestingly, DNA synthesis in the absence of OGP-(10–14) was marginally inhibited by Mapkapk2 siRNA, suggesting a possible role for this kinase in setting the basal level of cell proliferation.

OGP-(10–14) Stimulates Long Term CREB Phosphorylation and Transcriptional Activity—Because CREB is one of the main targets of Mapkapk2 (29), we assessed the effect of OGP-(10–14) on CREB activation. Although CREB is constitutively expressed in MC3T3 E1 cells, CREB phosphorylation is normally low, becomes readily detectable within 1 h after stimulation, and returns to a low level thereafter (30). Indeed, phosphorylated CREB was readily detectable in cultures 30 min after medium change; however, OGP-(10–14) had no effect on CREB phosphorylation at this early time point (Fig. 5A). By contrast, although CREB phosphorylation returned to the low, hardly detectable levels in the control cultures 8 h after medium change, it was sustained for at least 8 h after administration of medium containing OGP-(10–14) (Fig. 5A). Furthermore, this long term CREB phosphorylation was no longer observed in the presence of PD098059, suggesting the involvement of the MEK-ERK1/2-Mapkapk2 axis.

Luciferase assays were then performed to functionally investigate the transcriptional outcome after stimulation of the ERK1/2-Mapkapk2-CREB axis with OGP-(10–14). The effect of the peptide at various concentrations was measured in MC3T3 E1 cells stably transfected with a luciferase construct that reports on CREB transcriptional activity. As shown in Fig. 5B, OGP-(10–14) had a biphasic effect on luciferase activity in the MC3T3 E1/CRE-luc cells, with the maximum stimulation observed at 10–14 M. This bell-shaped curve is reminiscent of the OGP-(10–14) dose-response curve observed when DNA synthesis is measured (14). The OGP-(10–14) stimulation of CREB transcriptional activ-
ity was mitigated dose-dependently by the MEK-ERK1/2 inhibitor PD098059 (Fig. 5C) and by Mapkapk2 siRNA (Fig. 5D). Jointly, the established relationship between Mapkapk2 and CREB, the delayed OGP-(10–14) activation of both proteins, and the attenuation of the OGP-(10–14)-induced CREB activation and transcriptional activity by the MEK-ERK1/2 inhibitor PD098059 and Mapkapk2 siRNA (Fig. 5) suggest that CREB is the downstream link in an OGP-(10–14)-activated mitogenic signaling axis that is dependent on the stimulation of ERK1/2 activity and de novo synthesis of Mapkapk2.

**DISCUSSION**

In this study we show that the OGP-(10–14) mitogenic signaling in osteoblastic cells involves rapid phosphorylation of ERK1/2 and de novo Mapkapk2 mRNA and protein synthesis. Downstream of the activated Mapkapk2, OGP-(10–14) stimulates CREB phosphorylation and transcriptional activity (Fig. 6), which is inhibitable by suppressing ERK1/2 activation or Mapkapk2 protein synthesis.

Using the MC3T3 E1 cell line, we have reported recently (17) that OGP-(10–14) acutely stimulates MAP kinase activity. ERK1/2 have been reported previously to mediate a handful of extracellular signals in osteoblasts (31–34). Therefore, to identify the specific MAP kinases activated by OGP-(10–14), we initially asked whether the peptide stimulates ERK1/2 phosphorylation. Indeed, OGP-(10–14) induced rapid and potent stimulation of ERK1/2 phosphorylation. Moreover, this stimulation and the OGP-(10–14) mitogenic effect were blocked by the MEK (ERK1/2-activating kinase) inhibitor PD098059, indicating that ERK1/2 activation is a critical link in the OGP-(10–14) stimulation of DNA synthesis. Studies in osteoblastic and other cells demonstrated acute (minutes to hours) and/or delayed (days) ERK1/2 activation in response to different extracellular stimuli. Rapid responses that are sustained for several days were also reported (35, 36). The acute response is attributable to direct, primary receptor-triggered phosphorylation events, whereas the delayed ERK1/2 activation is thought to be preceded by events such as calcium mobilization, prostaglandin production, and protein synthesis (37, 38). The results presented here suggest that the OGP-(10–14) mitogenic effect is dependent, at the very least in part, on rapid ERK1/2 activation. This is based on the following observations: (i) ERK1/2 was activated after a 5–60-min challenge with OGP-(10–14), and this activation was prevented by 15 min of treatment with PD098059; (ii) the PD098059 suppressed OGP-(10–14)-stimulated Mapkapk2 mRNA level after 4 h; and (iii) the PD098059 inhibited OGP-(10–14)-induced CREB phosphorylation after 8 h.

We have noted previously that unlike the case of many other mitogens such as platelet-derived growth factor and fibroblast growth factor-2 (whereby stimulation of DNA synthesis is measurable already after 24 h) (39, 40), ~48 h are required before the mitogenic action of OGP-(10–14) becomes traceable (10). We therefore assumed that the OGP-(10–14)-activated signaling cascade downstream of the MAP kinase involves de novo mRNA and protein synthesis. Indeed, using hybridization to cDNA arrays, real-time RT-PCR, and Western analyses we demonstrate that accumulation of Mapkapk2 mRNA and protein are critical events in the OGP-(10–14) mitogenic signaling. We further show a parallel increase in phosphorylated Mapkapk2, suggesting that stimulation of the non-phosphorylated Mapkapk2 substrate leads to a parallel stimulation of the phosphorylated Mapkapk2 product. The increase in activated Mapkapk2 is traceable after several hours but not within the first hour of challenge with OGP-(10–14), in line with the requirement for de novo protein synthesis. That de novo Mapkapk2 protein synthesis is essential for the OGP-(10–14) mitogenic activity is demonstrated by its inhibition using Mapkapk2 siRNA. Although Mapkap2, also designated MK2 (22), is generally thought to be a substrate for p38 (41, 42), it was originally identified as an ERK target (43). Furthermore, PD098059 partially inhibits the activation of Mapkapk2 by the G-protein coupled receptor agonist endothelin-1 (44). Although it is not clear to what extent the ERK stimulation of Mapkapk2 is dependent on de novo protein synthesis, such dependence is suggested by several instances where prolonged challenging was required for its activation (45, 46). That the OGP-(10–14) stimulation of Mapkapk2 synthesis and phosphorylation is dependent on ERK1/2 activation is shown by its complete inhibition with PD098059. The other MAP kinase candidate that we tested, p38, is not activated by exposure to OGP-(10–14), and the p38 inhibitor SB203580 does not affect the OGP-(10–14)-stimulated DNA synthesis suggesting that ERK1/2 are the only MAP kinases involved in the OGP-(10–14) mitogenic signaling.

CREB is one of the best characterized stimulus-induced transcription factors. It activates transcription of target genes in response to a diverse array of stimuli, including peptide hormones and growth factors that stimulate a variety of protein kinases (47). In osteoblastic cells CREB is activated by extracellular stimuli including parathyroid hormone, epidermal growth factor, and prostaglandin E2 (7, 48). Furthermore, a parathyroid hormone-activated pathway involving ERKs and CREB has been implicated recently (31) in osteoblasts. The present data demonstrate an osteoblastic mitogenic pathway involving transcriptional activity by CREB. These data further suggest that Mapkapk2 links the activation of ERK1/2 and CREB, thus leading to increased DNA synthesis.

It has been demonstrated previously in the MC3T3 E1 osteoblastic cell line and primary stromal cells derived from adult human bone marrow that the activity of OGP is concentration-dependent (9, 49–51). At low doses, exogenous OGP stimulates endogenous OGP production with the resultant increase in exogenous OGP stimulating mitogenesis (10). The fast increase in endogenous OGP production is not attributable to de novo protein synthesis. Hence, it is most likely that the pathway proposed by our previous (17) and present results, which involves the stimulation of Mapkapk2 mRNA and protein synthesis, is employed by the osteoblastic mitogenic response proper. In the MC3T3 E1 model this pathway consists of G protein and ERK1/2 activation, accumulation of Mapkapk2, and stimulation of CREB (Fig. 6). Moreover, the similarity in the OGP-(10–14)-induced ERK1/2 and Mapkapk2 activation between the MC3T3 E1 cell line and the primary NeMCO model suggests that this pathway is shared by osteoblastic cells in vivo. Further investigation is needed to validate this pathway in human osteoblasts and to identify missing links upstream of Mapkapk2 and downstream of CREB.

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