Protein Kinase C-independent Activation of Protein Kinase D Is Involved in BMP-2-induced Activation of Stress Mitogen-activated Protein Kinases JNK and p38 and Osteoblastic Cell Differentiation*

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An important role for JNK* and p38 has recently been discovered in the differentiating effect of bone morphogenetic protein 2 (BMP-2) on osteoblastic cells. In this study, we investigated the molecular mechanism by which BMP-2 activates JNK and p38 in MC3T3-E1 osteoblastic cells. Activation of JNK and p38 induced by BMP-2 was blocked by the protein kinase C/protein kinase D (PKC/PKD) inhibitor Go6976 but not by the related compound, Go6983, a selective inhibitor of conventional PKCs. Associated with this inhibitory effect of Go6976, BMP-2 induced a selective and a dose-dependent Ser916 phosphorylation/activation of PKD, which was also blocked by Go6976. In contrast to the recently described PKC-dependent molecular mechanism involved in activation of PKD by G protein-coupled receptor agonists, BMP-2 did not induce a phosphorylation of PKD on Ser447/448. To further document an implication of PKD in activation of JNK and p38 induced by BMP-2, we constructed MC3T3-E1 cells stably expressing PKD antisense oligonucleotide (AS-PKD). In AS-PKD clones having low PKD levels, activation of JNK and p38 by BMP-2, but not of Smad1/5, was markedly impaired compared with empty vector transfected (V-PKD) cells. Analysis of osteoblastic cell differentiation in AS-PKD compared with V-PKD cells showed that mRNA and protein expressions of alkaline phosphatase and osteocalcin induced by BMP-2 were markedly reduced in AS-PKD. In conclusion, results presented in this study indicate that BMP-2 can induce activation of PKD in osteoblastic cells by a PKC-independent mechanism and that this kinase is involved in activation of JNK and p38 induced by BMP-2. Thus, this pathway, in addition to Smads, appears to be essential for the effect of BMP-2 on osteoblastic cell differentiation.

BMPs† are members of the TGF-β superfamily and exert a wide range of biological effects in different tissues. In particular, they contribute to the formation of bone and connective tissues (1) by inducing the differentiation of mesenchymal cells into bone-forming cells (2). BMP-2 expression has been observed in a large variety of cells including osteoblasts (3). This factor is one of the most potent stimulators of osteoblastic cell differentiation, which is mainly characterized by expression of ALP, type I collagen, and Oc (4–6). Members of the TGF-β superfamily exert their biological activities by binding to cell surface type I and II serine/threonine kinase receptors. The type II receptor phosphorylates type I receptor, which in turn phosphorylates and activates intracellular substrates such as proteins of the Smad family. Smad 1 (7), and the closely related Smads 5 and 8, specifically mediate BMP-2 responses, such as, for example, the osteoblastic differentiation of precursor cell lines (8, 9). Upon phosphorylation, Smad 1/5/8 proteins interact with a common partner, Smad 4, and the complex Smad 1/5/8-Smad 4 translocates to the nucleus where it exerts transcriptional activity either through direct binding to DNA or via association with other DNA-binding proteins (10). Among signal transducers recently reported to participate in TGF-β signaling, the MAPKs probably play a significant role in cooperating with Smads. MAPKs are a group of well described serine/threonine kinases implicated in the transmission of extracellular signals to intracellular targets (11). Cooperative interaction between Smads and transcription factors activated by MAPKs has been recently described for TGF-β signaling. Several TGF-β-responsive elements containing AP1 binding sites are activated by c-Jun/c-Fos heterodimers (12). It has also been reported that TGFβ and Smad 3 only weakly induce AP1-containing promoters in absence of c-Jun or c-Fos binding suggesting that Smads require active c-Jun/c-Fos dimers as DNA binding partners (13). Interestingly, Smads were found to preferentially interact with the phosphorylated form of c-Jun (13), which is generated by the activity of JNK. As for c-Jun/c-Fos, Smads can also cooperate with activated ATF2, which is generated upon phosphorylation by p38 MAPK (14). In relation with these recent observations, we recently found that JNK and p38 are activated by BMP-2 and documented their implication in the differentiating effect of this bone morphogenetic protein in MC3T3-E1 and primary calvaria-derived osteoblastic cells (15). The molecular mechanism by which BMP-2 induces activation of JNK and p38 remains completely unknown. Recent studies indicated that BMP-2 can either activate the PI3 kinase Akt pathway in 2T3 cells (16) or PKC pathways in human neonatal calvaria cells (17). The protein kinases C (PKCs) comprise a family of intracellular serine/threonine spe-

coupled receptor; MAPK, mitogen-activated protein kinase; MEK, MAPK kinase; Oc, osteocalcin; PH, Pleckstrin homology; PLC, phospholipase C; PKD, protein kinase D; PGF2α, prostaglandin F2α; PMA, phorbol 12-myristate 13-acetate; TGFβ, transforming growth factor β; PKC, protein kinase C.
cific kinases, that are implicated in signal transduction of a wide range of biological responses, including changes in cell morphology, proliferation and differentiation (18–20). The 13 members of the family can be grouped into three major classes of Ca2+-dependent classical PKCs (cPKCs), Ca2+-independent, novel PKCs (nPKCs), and Ca2+ - and lipid-independent atypical PKCs (aPKCs). The fourth PKC subgroup, which consists of PKCμ (21), its mouse homologue protein kinase D1 (PKD) (22), PKCθ (23), and PKD2 (24), share common structures such as N-terminal cysteine fingers defining the structural basis for lipid-mediated activation. They differ from the three major groups of PKC isozymes by the presence of an acidic domain (25), a PH domain and the lack of a typical pseudosubstrate site. PKD is ubiquitously expressed and involved in diverse cellular functions (26–28), such as constitutive transport processes in epithelial cells (29), G protein-mediated regulation of Golgi organization (30) and protection from apoptosis (31).

Given the important role of the stress kinases JNK and p38 in BMP-2-induced osteoblastic cell differentiation (15), we sought to investigate the molecular mechanism involved in the activation of these MAP kinases and studied the role of PKCs in mediating this signaling process. We found that activation of PKD by a PKC-independent mechanism is involved in activation of these stress MAP kinases by BMP-2 in MC3T3-E1 cells. Using antisense oligonucleotide technique, we also provide compelling evidences that this signaling pathway is required for osteoblastic cell differentiation.

**EXPERIMENTAL PROCEDURES**

**Reagents, Antibodies, and Plasmids—**FCS, glutamine, antibiotics, and trypsin/EDTA were obtained from Invitrogen. n-MEM was purchased from Amino (Bioconcept, Allschwill, Switzerland) and FMA from Sigma (Sigma Chemical Co.). GST-c-Jun and GST-c-ATF2 were obtained from Santa Cruz Biotechnology Inc (Santa Cruz, CA). Go6976 and Go6983 were obtained from Calbiochem-Novabiochem Corp. (San Diego, CA). γ-32P]ATP was purchased from Amersham Biosciences.

Polyclonal anti-JNK, anti-p38, anti-PKCα/PKD (C terminus of mouse origin), and anti-p-c-Jun were obtained from Santa Cruz Biotechnology Inc (Santa Cruz, CA). Polyclonal anti-Smad 1/5 and anti-PKCe were from Upstate Biotechnology (Lake Placid, NY). Polyclonal anti-p38, anti-pPKD/PKCα (Ser286), anti-pPKD/PKCα (Ser287/288), pSmad 1/5, anti-pMEK4, anti-pMEK3.6, anti-pPan-PKC, anti-pPKDα, and the monoclonal anti-pJNK were obtained from New England BioLabs (Cell Signaling Technology, MA).

Cell Culture—Transfection—Mouse calvaria-derived MC3T3-E1 cells were cultured in α-MEM containing 10% fetal calf serum (v/v), 0.5% nonessential amino acids (v/v), 100 IU/ml penicillin, and 100 μg/ml streptomycin. In experiments aimed at testing the effect of BMP-2, cells were switched to 2% fetal calf serum 24 h before and during the study. When the influence of PKC inhibitors was investigated, agents were added 1 h prior to and during the experiments. For the construction of PKD antisense (AS-PKD) plasmid, an oligonucleotide of 70 bases, targeted against sequences adjacent to the ATG initiation codon of PKD (PKCδ) mRNA, was synthesized with BamHI and EcoRI sites in 5’ and 3’, respectively (MWG Biotech, Germany). The oligonucleotide sequence was 5’-GGGCT AGGCGGTCCGCAGCGTGAGGGACGCCATCACCCCGCAGTGGACCCCAAGA GTTGGCCGAGGCATGGG-3’. The oligonucleotide and its complementary strand were annealed and ligated into the mammalian expression vector pcDNA3.1 (Invitrogen) containing cytomegalovirus promoter and neomycin resistance gene. MC3T3-E1 cells were transfected with either the AS-PKD or sense gene. MC3T3-E1 cells were transfected with either the AS-PKD or sense gene with activation of Smads and the underlying molecular mechanism of activation remained to be investigated. Among functional signaling pathways recently described to mediate BMP-2 effects in osteoblastic cells, PKCs have been reported to be involved in BMP-2-induced apoptosis in human neonatal calvaria cells (17). To study the possible implication of a PKC in BMP-2-induced activation of stress MAP kinases JNK and p38, we used two staurosporine-derived selective PKC inhibitors, Go6983 and Go6976, that have different PKC inhibitory specificities. The former inhibits the activity of PKCα, PKCβI, PKCβII, PKCδ, and pKCy whereas the latter blunts PKCo and PKCβ as well as PKCα/PKCδ activities (37). As shown in Fig. 1, Go6983 had no effect on stress MAP kinases activation induced by BMP-2 whereas Go6976 completely blunted this response.

RESULTS

We recently found that BMP-2 can induce a stimulation of JNK and p38 in MC3T3-E1 and primary calvaria-derived osteoblastic cells (15). Expression of this newly described BMP-2-induced signaling pathway was found to be delayed compared with activation of Smads and the underlying molecular mechanism of activation remained to be investigated. Among functional signaling pathways recently described to mediate BMP-2 effects in osteoblastic cells, PKCs have been reported to be involved in BMP-2-induced apoptosis in human neonatal calvaria cells (17). To study the possible implication of a PKC in BMP-2-induced activation of stress MAP kinases JNK and p38, we used two staurosporine-derived selective PKC inhibitors, Go6983 and Go6976, that have different PKC inhibitory specificities. The former inhibits the activity of PKCα, PKCβI, PKCβII, PKCδ, and pKCy whereas the latter blunts PKCo and PKCβ as well as PKCα/PKCδ activities (37). As shown in Fig. 1, Go6983 had no effect on stress MAP kinases activation induced by BMP-2 whereas Go6976 completely blunted this response.
PKD Is Involved in BMP-2 Activation of JNK and p38

PKD is involved in BMP-2 activation of JNK and p38. Confluent MC3T3-E1 cells were preincubated with 10 μM of either Go6976 or Go6983 or their vehicle for 1 h and then exposed to BMP-2 (100 ng/ml) for 3 h. At the end of the incubation period, cells were rapidly frozen in liquid nitrogen before lysis at 4 °C. Lysates were then immunoprecipitated with specific anti-phospho-MAPK antibodies. Immune complexes were incubated with [γ-32P]ATP and the appropriate MAPK substrate in a phosphorylation buffer as described under “Experimental Procedures.” Following in vitro phosphorylation, proteins were heated in SDS sample buffer, subjected to SDS-PAGE electrophoresis, transferred to Immobilon P membranes and autoradiographed. Total proteins were investigated by Western blot analysis.

Inhibiting PKCs is PKC/PKD, this observation suggested that this kinase might be involved in BMP-2-induced activation of JNK and p38. To investigate whether PKD is involved in this signaling response, we first analyzed whether BMP-2 can activate PKD in MC3T3-E1 cells. Using a rabbit polyclonal antibody recognizing a C-terminal epitope, PKD appeared as a doubled protein band (~110 and 115 kDa) on Western blots (Fig. 2). Whether these two bands correspond to different members of the PKD family or of different sizes of one member is not clear at the moment. Of interest and as further discussed below, we found that BMP-2 mainly induced an increase in Ser916 phosphorylation of the higher molecular size of this doubled protein band and this effect was blunted by Go6976 but not by Go6983 and was still detectable in cells preincubated for 18 h with 1 μM PMA to down-regulate DAG-sensitive PKCs (Fig. 2). Associated with this effect of BMP-2 on Ser916 PKD phosphorylation, which was previously reported to modify the conformation of the kinase and to influence the duration of its kinase activity (38), we did not find any change in activation of either conventional PKCs or of PKCε and PKCα (Fig. 2), two novel PKCs recently shown to preferentially activate PKD (39).

Thus, this series of observations indicate that BMP-2 can activate PKD in osteoblast-like cells but the implication of a PKC inhibitor on activation of PKD and JNK and p38. BMP-2 was also performed and data are shown in Fig. 4. Clearly, changes in Ser916 phosphorylation of PKD induced by BMP-2 preceded by about 1 h the stimulation of c-Jun phosphorylation that correlates with activation of JNK in MC3T3-E1 cells (15). In this series of experiments, we also analyzed whether BMP-2 induces a change in PKC-mediated PKD Ser447/467 phosphorylation as recently reported in response to GPCR agonists in fibroblasts and epithelial cells (39, 40). In contrast to the reproducible effect of PGF2α on PKD Ser916 phosphorylation that correlates with activation of JNK in MC3T3-E1 cells (15), in this series of experiments, we never detected such effect in response to BMP-2 (Fig. 4B).

To further document that PKD is implicated in BMP-2-induced activation of JNK and p38, we constructed MC3T3-E1 cell lines stably expressing a PKD antisense oligonucleotide. The sequence of this oligonucleotide is described under “Experimental Procedures” and was targeted against the first ATG start codon of mouse PKD1 mRNA transcript. Expression of PKD was monitored in several clones and two of them (AS1-PKD and AS2-PKD) having selectively lost expression of the BMP-2-dependent activation of JNK and p38..
upper PKD protein band (Fig. 5A) were selected for further analysis. Clearly, the absence of this PKD protein band, which corresponds to the band in which we detected a change in the phosphorylation of PKD on Ser916 in response to BMP-2, was associated with a nearly complete loss of JNK and p38 activation induced by BMP-2 compared with a normal response in empty vector stably transfected cells (Fig. 5B). As expected, associated with the absence of this PKD molecule, basal and stimulated PKD activity as well as increase in Ser916 PKD induced by BMP-2 were markedly decreased (Fig. 6A). Of interest, activation of Smad1/5 by BMP-2 was practically normal in these AS-PKD clones (Fig. 6A and similar results for AS2 not shown) indicating that this effect is not related to impairment in BMP-2 receptor expression and/or activity.

To gain further insight into the molecular mechanism by which PKD induces activation of MAP kinases, we determined changes in MEK4 and MEK3,6 activities, which are upstream activators of JNK and p38, respectively. As shown in Fig. 6B, BMP-2 also induced the activation of these MEKs and this effect was blunted in AS-PKD compared with empty vector-transfected cells suggesting that PKD is probably influencing either the regulation or the activity of a MEK kinase.

Finally, we investigated whether this new signaling pathway induced by BMP-2 involving PKD activation of stress MAP kinases JNK and p38 plays a physiological role in osteoblastic cells. For this analysis, two markers of osteoblastic cell differentiation, ALP and Oc were investigated. As expected from our recent observation that p38 and JNK are implicated in BMP-2-induced stimulation of ALP and Oc (15), the biochemical (Fig. 7, A and B) and mRNA (Fig. 7C) expression of these proteins were markedly reduced in AS-PKD compared with pcDNA3-transfected cells.

**DISCUSSION**

In our laboratory, we recently found that the differentiating effect of BMP-2 on osteoblastic cells, not only requires the Smads, but also involves activation of the stress JNK and p38 pathways (15). This finding agrees with the recent observation that members of the AP1 family of transcription factors, which are activated by MAP kinases, are DNA binding partners for Smads (13). This information strongly suggests that protein complexes between members of these two families are playing an important role in regulating osteoblastic cell differentiation.

Kinetic analysis of JNK and p38 activation induced by BMP-2 in MC3T3-E1 cells indicated that this signaling re-
PKD Is Involved in BMP-2 Activation of JNK and p38

Fig. 7. Implication of PKD in BMP-2-induced osteoblastic cell differentiation. A, empty vector (pcDNA) or PKD antisense oligonucleotide (AS1) stably transfected cells were treated for 48 h with BMP-2 (100 ng/ml) and alkaline phosphatase activity (ALP) was determined as described under “Experimental Procedures.” B, the same cells were treated for 5 days with BMP-2 (100 ng/ml) before analysis of osteocalcin (Oc) production as described under “Experimental Procedures.” C, cells were also treated for 48 h with BMP-2 (100 ng/ml) before Northern blotting analysis as described under “Experimental Procedures.” Data shown in A and B are mean ± S.E. of four determinations from a representative experiment. *, p < 0.01, significantly different from the respective control.

To validate the implication of PKD in activation of JNK and p38 induced by BMP-2, we constructed MC3T3-E1 cells having a low PKD level using an antisense oligonucleotide experimentally approached described under “Experimental Procedures.” Interestingly, the stable expression of an antisense oligonucleotide, targeted against a nucleotide stretch encompassing the first ATG start codon of PKD1 mRNA, selectively eliminated the high molecular weight protein of the doubled PKD bands (Fig. 5A), which is precisely the protein that is phosphorylated on Ser916 in response to BMP-2 (Figs. 2 and 6). To investigate whether BMP-2 can activate PKD, data shown in Figs. 2–4 clearly indicate that BMP-2 induces an increase in Ser916 PKD phosphorylation, which is associated with activated autokinase activity (Figs. 3A and 6A). In MC3T3-E1 cells, we found that the specific anti-C-terminal PKD polyclonal antibody detects two protein bands with ~110–115 kDa molecular size in Western blotting. Whether these two bands represent two protein bands with respective control. BMP-2 (Fig. 2). Collectively, this first series of observations strongly suggested that Ser916 phosphorylation/activity of PKD induced by BMP-2 is implicated in the stimulation of stress MAP kinases in osteoblastic cells. Several mechanisms have been described for in vivo activation of PKD, essentially from PKD1 analysis. Activation of PLC, leading to production of DAG and thereby activation of PKCζ and/or PKCγ which in turn phosphorylates PKD1 in the activation loop is the best documented mechanism (39, 42–44). Recently, it has been shown that this activation occurs through the release of an autoinhibitory mechanism (45). Apart from this PLC-DAG-PKC-dependent activation of PKD, it has been suggested that Gβγ subunits can activate PKD1 through direct interaction with the PH domain (30). A third mechanism is through caspase-mediated cleavage during the induction of apoptosis by genotoxic drugs (46). Tyrosine phosphorylation of PKD is the last mechanism described for activation of this kinase. The molecular mechanism by which tyrosine phosphorylation influences PKD activity also involves a release of the autoinhibitory effect of the PH domain (47). In MC3T3-E1 cells, Ser916 phosphorylation/activity of PKD induced by BMP-2 was neither associated with PKD phosphorylation of Ser744/748 (Fig. 4B) nor with either a cleavage of PKD or a change in tyrosine phosphorylation (data not shown). The enhanced Ser916 phosphorylation of PKD induced by PKD was detected in cells pretreated for 24 h with phorbolesters that induced a complete down-regulation of DAG-dependent PKCs (Fig. 2) further suggesting that a PKC-dependent mechanism is not involved in activation of PKD in response to BMP-2. In contrast with the PKC-independent activation of PKD induced by BMP-2, we found that PGF2α, which acts through a GPCR in MC3T3-E1 cells, induces a rapid translocation of PKD from a soluble to a particulate fraction, a response associated with enhanced PKD phosphorylation on Ser744/748 (Fig. 4B). The enhanced phosphorylation of PKD on Ser744/748 induced by PGF2α was completely blocked by Go6983 (data not shown, manuscript in preparation) suggesting a PLC-DAG-PKC-dependent mechanism of PKD activation by GPCR agonists in MC3T3-E1 cells as reported in other cell systems (39, 42–44). This observation strongly suggests the coexistence of two different PKC-dependent and -independent cellular mechanisms for activation of PKD in MC3T3-E1 osteoblast-like cells. The molecular mechanism involved in PKD activation by BMP-2 remains to be investigated. Since activation of PKD by BMP-2 is a delayed process (Fig. 4), it may involve the production of a regulatory protein. Theoretically, the binding of such a putative regulatory protein to the PH domain could, as mentioned above, modulate the activity of PKD.
MEKK, an interesting issue that remains to be investigated. Finally, as expected from our recent observation that JNK and p38 are important signaling pathways for mediating the differentiating effect of BMP-2 (15), we found that impairment of JNK and p38 activation by BMP-2 in cells having a low PKD level is associated with a nearly complete blunting of BMP-2-induced cell differentiation (Fig. 7, A and B), despite a normal activation of Smads by this bone morphogenetic protein (Fig. 5A). As recently discussed in our above mentioned study, the molecular mechanism by which JNK and p38 mediate the effect of BMP-2 on cell differentiation is not known but probably involves a transcriptional process (15), a working hypothesis that is confirmed in data shown in Fig. 7C indicating that associated with the blunted expression of ALP and Oc, corresponding mRNA changes of these two markers of osteoblastic cell differentiation was also impaired in low PKD-expressing cells.

In conclusion, data reported in this study indicate that PKD is implicated in the stimulation of JNK and p38 induced by BMP-2 in osteoblastic cells, which are MAP kinase pathways required for optimal cell differentiation induced by this bone morphogenetic protein. They also unravel the existence of a new PKC-independent molecular mechanism of PKD activation that remains to be investigated.

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