Specific Activation of the p38 Mitogen-activated Protein Kinase Signaling Pathway and Induction of Neurite Outgrowth in PC12 Cells by Bone Morphogenetic Protein-2*

(Received for publication, January 26, 1999, and in revised form, May 18, 1999)

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Bone morphogenetic protein (BMP)-2 has the capacity to induce the neuronal differentiation of PC12 cells. Unlike nerve growth factor, however, BMP-2 failed to induce the activation of the 41-/43-kDa mitogen-activated protein (MAP) kinase pathway in these cells. In contrast, BMP-2 characterizedly induced the sustained activation of the p38 MAP kinase pathway. Pretreatment of PC12 cells with SB203580 inhibited the BMP-2-induced neurite outgrowth formation in a dose-dependent manner; this inhibition coincided well with the ability of SB203580 to inhibit the BMP-2-induced activation of the p38 MAP kinase pathway. Overexpression in PC12 cells of wild-type MAP kinase kinase (MKK)-6 enhanced the BMP-2-induced activation of p38 MAP kinase, whose activation correlated well with the ability of these cells to induce neurite outgrowth in response to BMP-2. Transient expression of kinase-negative forms of MKK3/6 inhibited the formation of neurite outgrowth in response to BMP-2. Furthermore, expression of constitutively active forms of MKK3/6 induced neurite outgrowth without BMP-2 stimulation, and SB203580 inhibited this induction. These results clearly indicate that activation of the p38 MAP kinase pathway is necessary for BMP-2-induced neuronal differentiation of PC12 cells. Our results also suggest that activation of the p38 MAP kinase pathway alone can induce the neuronal differentiation of PC12 cells.

Bone morphogenetic proteins (BMPs),1 members of the transforming growth factor-β superfamily, were originally identified by their novel activity to induce cartilage and bone formation in ectopic extraskeletal sites in vivo (1, 2). A number of studies have demonstrated that BMPs play essential roles in bone formation and bone cell differentiation; e.g. BMPs stimulate proteoglycan synthesis, alkaline phosphatase activity, collagen synthesis, and osteocalcin expression in chondroblasts/osteoblasts (3–5). However, expression of BMPs (6–8) and their receptors (9) in many tissues other than bone suggests that they are also involved in the regulation of many biological processes unrelated to bone formation. In accordance with this possibility, increasing evidence suggests a regulatory role for BMPs in early vertebrate embryogenesis such as in mesoderm induction, limb development, and hematopoietic formation (10–14). Moreover, BMPs have drawn attention as possible regulators of central nervous system development; BMPs are expressed in multiple central nervous system regions throughout development (15–17). In this respect, we have recently demonstrated that BMP-2 acts as a neurotrophic factor; BMP-2 induces the neuronal differentiation of rat pheochromocytoma PC12 cells (18) and also promotes the survival and differentiation of striatal GABAergic neurons in culture (19).

BMPs transduce their signals by binding to two different types of serine/threonine kinase receptor, type I and type II (20–22). Upon ligand binding, type I and type II receptors form heteromeric receptor complexes. Then type I receptors are phosphorylated by type II receptors, and subsequent activation of the catalytic activity of type I receptor kinase is essential for BMP signaling. Although phosphorylation of Smad proteins by the activated type I receptor kinase was suggested to play an important role in the mediation of BMP signaling (20–22), the precise cytoplasmic signaling pathway for BMPs through which it elicits its effects on the regulation of wide variety of biological processes remains largely unknown.

We have recently shown that BMP-2 has the capacity to induce the neuronal differentiation of PC12 cells. Unlike NGF, however, BMP-2 did not induce the activation of MEK or 41-/43-kDa MAP kinases (ERK2 and ERK1, respectively) in these cells (18). MEK and ERK1/2 constitute a protein kinase cascade (the ERK cascade), which is one of the major cytoplasmic signaling pathways commonly activated in a wide variety of cells by diverse extracellular stimuli (23–26). Activation of the cascade elicits a wide array of physiological responses such as cell division, differentiation, and secretion (23–26), and it has been reported that the activation of the cascade is necessary and sufficient for PC12 cell neuronal differentiation (27, 28). However, our previous findings, which showed a clear distinction between the requirement for the activation of MEK and ERK1/2 and the ability of BMP-2 to induce PC12 cell neuronal differentiation (18), have indicated that the activation of the ERK cascade is not an absolute requirement for PC12 cell differentiation. In fact, our results suggested the existence of alternative signaling pathway(s) that would be essential for...
eliciting the neurotrophic effect of BMP-2.

In the present study, we have investigated the possible involvement of other members of the MAP kinase family, p38 MAP kinase and JNK, in the BMP-2 signaling pathway that leads to the induction of neuronal differentiation of PC12 cells. For the analysis, we utilized a synthetic inhibitor of p38 MAP kinase and vectors for the expression of kinase-negative/constitutively active forms of MK3/6. Our results demonstrate that activation of the p38 MAP kinase signaling pathway is required for the BMP-2-induced neuronal differentiation of PC12 cells.

EXPERIMENTAL PROCEDURES

Materials—Recombinant human BMP-2, expressed in silkworm larvae, was homogenized as described previously (28). Purified human activin A was kindly provided by Dr. Kaoru Miyamoto (National Cardiovascular Center, Research Institute, Osaka, Japan). β-NGF purified from mouse submaxillary glands was purchased from Toyobo Co. (Osaka, Japan). A polyclonal anti-ERK antibody was raised against residues 299–321 (RIEVQALHYPLEQYYDPSDEP) of 41-kDa MAP kinase (ERK2) and was shown to recognize both ERK1 and ERK2 (18, 30, 31). The polyclonal anti-p38 MAP kinase antibody (C-20) was obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). The monoclonal anti-JNK1 antibody (G151–333.8) was purchased from Pharmingen; the polyclonal anti-MAPKAP kinase-2 antibody and MAPKAP kinase-2 substrate peptide were from Upstate Biotechnology, Inc.; and the polyclonal anti-active (phosphorylated) ERK antibody and the polyclonal anti-active (phosphorylated) p38 MAP kinase antibody were from Promega. The monoclonal anti-HA antibody (12CA5) and monoclonal anti-c-Myc antibody (9E10) were purchased from Roche Molecular Biochemicals; the monoclonal anti-FLAG antibody (M2) was from Sigma; and the FITC-conjugated goat anti-mouse IgG antibody (doubling staining grade) was from Zymed Laboratories Inc. The cDNAs for GST-c-Jun-(1–79) (32) and GST-ATF2-(1–254) (kind gifts from Dr. Masahiko Hibi, Osaka University Medical School) were expressed in *Escherichia coli* as GST fusion proteins. GST fusion proteins were purified by affinity chromatography using GSH-agarose (Amersham Pharmacia Biotech). Briefly, each immunoprecipitate was incubated for 60 min at 30 °C with 20 μl of GST-c-Jun-(1–79), and MAPKAP kinase-2 assays were performed with 2 μg of GST-ATF2-(1–254) as substrate. JNK assays were performed using 2 μg of GST-c-Jun-(1–79), and MAPKAP kinase-2 assays were performed using a 100 μM concentration of the MAPKAP kinase-2.
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substrate peptide. For assays of p38 MAP kinase and JNK, reactions were terminated by adding 10 μl of 4× SDS sample buffer, and the incorporation of 32P into substrate proteins was examined by SDS-polyacrylamide gel electrophoresis followed by autoradiography using a Fuji Bioimaging analyzer BAS 1500 (Fuji Photo Film Co., Tokyo, Japan). For assays of MAPKAP kinase-2, reactions were terminated by adding 10 μl of 1% orthophosphoric acid containing 1 mM ATP and 1% bovine serum albumin. An aliquot (30 μl) was then spotted onto phosphocellulose filters (P81, Whatman), and radioactivity incorporated into the substrate peptide was determined by liquid scintillation spectrometry after washing the filters five times in 150 mM phosphoric acid.

ERK activity was measured in 10 μg of cell lysates using the BIO-TRAKTM p42/p44 MAP kinase enzyme assay system (Amersham Pharmacia Biotech).

Western Blot Analysis—Cell lysates were separated by SDS-polyacrylamide gel electrophoresis, electrophoretically transferred to an Immobilon-P membrane (Millipore Corp.), and subjected to immunodetection using the appropriate primary antibody and horseradish peroxidase-conjugated secondary antibody. Proteins were visualized with the Enhanced Chemiluminescence system (Amersham Pharmacia Biotech) (18, 31).

Plasmids and Transfection—The expression vectors for wild-type MKK3 (pRSV-FLAG-MKK3), a kinase-negative form of MKK3 (pRSV-FLAG-MKK3 (Ala)) and a constitutively active form of MKK3 (pRSV-FLAG-MKK3 (Glu)) (34) were kindly provided by Dr. Roger J. Davis (University of Massachusetts Medical School), that for wild-type MKK6 (pME-HA-MKK6) (35) was kindly provided by Dr. Masatoshi Hagiwara (Tokyo Medical and Dental University), and those for a kinase-negative form of MKK6 (pCS-Myc-MKK6 (Ala)); ATP-binding site lysine was replaced with alanine) and a constitutively active form of MKK6 (pCS-Myc-MKK6 (Glu)); phosphorylation sites Ser-207 and Thr-211 were replaced with glutamic acid) were kindly provided by Dr. Kenji Sugiyama (Nippon Boehringer Ingelheim Co., Ltd.) (2). Transfection of these plasmids into PC12 cells was done by the LipofectAMINE method according to the manufacturer's instructions (Life Technologies, Inc.), using 5 μg of DNA/60-mm dish unless otherwise indicated. For the generation of PC12 cells overexpressing wild-type MKK6, 1 μg of DNA of pME-HA-MKK6 was transfected into the cells together with 9 μg of DNA of pSV2neo. Cells were then selected for 8 days in the growth medium supplemented with 400 μg/ml of Geneticin (Life Technologies, Inc.), and pools of selected cultures were used in the studies. More than 90% of the selected cells were HA-positive as viewed by fluorescent microscopy using the anti-HA antibody and FITC-conjugated goat anti-mouse secondary antibody (data not shown).

RESULTS

BMP-2 Induces the Selective Activation of p38 MAP Kinase Signaling Pathway in PC12 Cells—BMP-2 treatment of PC12 cells induced their neuronal differentiation, as shown clearly by the appearance of neurite outgrowth (Fig. 1). Activin A, a member of the transforming growth factor-β superfamily, also induced the neuronal differentiation of PC12 cells, although less strongly compared with BMP-2. Unlike the NGF-induced neuronal differentiation of PC12 cells, however, activation of the 41/43-kDa MAP kinases (ERKs) was not detected at all in the BMP-2- or activin A-treated cells (Fig. 2A and data not shown; Ref. 18). We next examined the possible involvement of other members of the MAP kinase family, p38 MAP kinase and JNK, in the signaling pathway of BMP-2 and activin A that leads to the induction of neuronal differentiation of PC12 cells.

The activation of the p38 MAP kinase was determined by two different procedures: by measuring the appearance of the active form on immunoblots using an anti-active (phosphorylated) p38 MAP kinase antibody and by a direct in vitro kinase assay of the immunoprecipitates using GST-ATF2-(1–254) as substrate. These analyses always gave an essentially identical time course profile for p38 MAP kinase activation. As shown in Fig. 2A and B, BMP-2 induced a notable and sustained activation of p38 MAP kinase in PC12 cells. p38 MAP kinase activation reached the maximum level within 60 min and then declined gradually. However, a considerable level of activation could still be detected even 4 h after stimulation of the cells. Moreover, BMP-2 induced the activation of MAPKAP kinase-2, a downstream effector kinase of p38 MAP kinase (36), in PC12 cells with a time course profile similar to that of p38 MAP kinase activation (Fig. 3). Activin A also induced the activation of p38 MAP kinase and MAPKAP kinase-2 in PC12 cells with similar kinetics, but less efficiently compared with BMP-2-treated cells. In contrast, neither BMP-2 nor activin A induced any significant activation of JNK in PC12 cells (Fig. 2B and data not shown).

SB203580, a Specific Inhibitor of p38 MAP Kinase, Inhibits the BMP-2-induced Neuronal Differentiation of PC12 Cells—To examine the role of activation of the p38 MAP kinase signaling pathway in BMP-2-induced neuronal differentiation of PC12
cells, the cells were pretreated for 30 min with varying concentrations or 10 μM of SB203580, a specific inhibitor of p38 MAP kinase (37) and then stimulated with BMP-2. Because the inhibition of p38 MAP kinase by SB203580 is in a reversible manner in vivo that cannot be detected by in vitro immune complex kinase assays of p38 MAP kinase (38), its inhibition by SB203580 was measured by determining MAPKAP kinase-2 activity. As shown in Fig. 3, pretreatment of the cells with SB203580 inhibited the BMP-2-stimulated activity of MAPKAP kinase-2 in a dose-dependent manner, with almost complete inhibition being observed at 10 μM. Pretreatment of PC12 cells with other inhibitors such as wortmannin (a specific inhibitor of phosphatidylinositol 3-kinase) (39) or PD98059 (a specific inhibitor of MEK) (40) did not affect the BMP-2-induced differentiation of the cells; nor did these agents inhibit the BMP-2-induced activation of MAPKAP kinase-2 (data not shown).

Overexpression of Wild-type MKK6 in PC12 Cells Enhances p38 MAP Kinase Activation and Neurite Outgrowth Induction in Response to BMP-2—Activation of p38 MAP kinase is achieved by combined tyrosine and threonine phosphorylation catalyzed by dual specificity kinases, MKK3 and MKK6; MKK6 has been reported to be the major activator of p38 MAP kinase (23–26, 41). Thus, we next generated PC12 cells overexpressing wild-type MKK6, in order to investigate the causal link between the activation of the p38 MAP kinase pathway and the

 FIG. 3. Activation of MAPKAP kinase-2 in PC12 cells treated with BMP-2 or activin A and the inhibitory effect of SB203580 pretreatment on MAPKAP kinase-2 activation. PC12 cells were preincubated with 0.1% Me2SO or 10 μM SB203580 for 30 min, followed by stimulation with 30 ng/ml of BMP-2 for the indicated periods of time (left), or PC12 cells were preincubated with 0.1% Me2SO or various concentrations of SB203580 for 30 min, followed by stimulation with 30 ng/ml of BMP-2 or 30 ng/ml of activin A for 60 min (right). The MAPKAP kinase-2 assay was performed by incubating cell lysates (40 μg of protein) with anti-MAPKAP kinase-2 antibody followed by the kinase reaction, and radioactivity incorporated into MAPKAP kinase-2 substrate peptide was determined. Each value represents the mean ± S.D. of duplicate determinations of a representative experiment. Data shown are representative of three separate experiments that gave essentially the same results.

 FIG. 4. BMP-2/activin A-induced activation of p38 MAP kinase, JNK, or ERKs in PC12 cells overexpressing wild-type MKK6. PC12 cells were transfected with pSV2neo (neo) or co-transfected with pSV2neo and pME-HA-MKK6 (9:1) (neo + MKK6). Cells were then selected for 8 days in the growth medium supplemented with 400 μg/ml Geneticin. After incubating in the serum-free medium for 12 h, pools of the selected cultures were mock-treated (Control) or stimulated with 30 ng/ml BMP-2 or 30 ng/ml activin A for 60 min. The p38 MAP kinase (p38 MAPK), JNK, and ERK assays were performed as described under “Experimental Procedures” using 60, 60, or 10 μg of cell lysates, respectively. Each value represents the mean ± S.D. of duplicate determinations of a representative experiment. Data shown are representative of two separate experiments that gave essentially the same results.
neuronal differentiation of PC12 cells. Overexpression of MKK6 in PC12 cells induced a ~1.5-fold increase in the basal p38 MAP kinase activity compared with that observed in the pSV2neo-transfected cells (Fig. 4), and this increase was accompanied by the appearance of short processes (Fig. 5). Treatment of the MKK6-transfected PC12 cells with BMP-2 or activin A induced marked activation of p38 MAP kinase; the degree of activation was ~2-fold higher than that observed with the pSV2neo-transfected cells stimulated with either of these agents (Fig. 4). In contrast, overexpression of MKK6 in PC12 cells did not induce the activation of JNK or ERKs at all in response to BMP-2 or activin A (Fig. 4 and data not shown).

Importantly, overexpression of wild-type MKK6 enhanced not only the BMP-2/activin A-induced activation of p38 MAP kinase but also the ability of PC12 cells to induce neurite outgrowth in response to BMP-2 or activin A. The MKK6-transfected PC12 cells started to extend neurite outgrowth within the initial 12 h of BMP-2/activin A stimulation (data not shown). Treatment of the MKK6-transfected cells with BMP-2/activin A for 36 h induced the formation of long neurite outgrowths (Fig. 5), which were more prominent than those observed in the pSV2neo-transfected PC12 cells stimulated with BMP-2/activin A for more than 4 days (see Fig. 1). Furthermore, pretreatment of the MKK6-transfected PC12 cells with SB203580 (10 μM) inhibited the BMP-2/activin A-stimulated activity of MAPKAP kinase-2 (data not shown) as well as the ability of the cells to induce neurite outgrowth in response to BMP-2 or activin A (Fig. 5).

Expression of Kinase-negative Forms of MKK3/6 Inhibits BMP-2-induced Neurite Outgrowth, while Expression of Constitutively Active Forms of MKK3/6 Induces Neurite Outgrowth without BMP-2 stimulation in PC12 Cells—In order to further confirm the essential role of the p38 MAP kinase pathway in BMP-2-induced neurite outgrowth in PC12 cells, the cells were transiently transfected with an expression vector encoding the kinase-negative form of MKK3 or MKK6. The transfection efficiency of these cDNA into PC12 cells was ~10% in repeated experiments. Thus, the effect of exogenous expression of mutant forms of MKK3/MKK6 on the p38 MAP kinase activity could not be determined precisely in such a mixed cell population. For this reason, we only analyzed the morphological changes of cells in which expression of the respective cDNA was confirmed by immunofluorescent microscopy after treating the cell population with the corresponding anti-epitope antibody and FITC-conjugated goat anti-mouse secondary antibody.

Although expression of wild-type MKK3 by itself did not affect the morphology of PC12 cells significantly, it enhanced the ability of cells to induce neurite outgrowth in response to BMP-2, as was the case for the overexpression of wild-type MKK6 in the cells (Fig. 5). In contrast, expression of the kinase-negative form of either MKK3 or MKK6 apparently inhibited the formation of neurite outgrowth in response to BMP-2 or activin A (Fig. 6, and data not shown). Furthermore, co-transfection into PC12 cells of kinase-negative forms of both MKK3 and MKK6 blocked the BMP-2-induced neurite outgrowth to a greater extent than that observed in cells expressing either of them alone; both of the anti-epitope antibodies used were of mouse origin, and thus we could not confirm whether or not each of the cells examined actually expressed both cDNAs.

Finally, PC12 cells were transiently transfected with an expression vector encoding the constitutively active form of MKK3 or MKK6. As shown in Fig. 7, expression of either of these constitutively active kinases clearly induced neurite outgrowth in PC12 cells in the absence of BMP-2 stimulation. The neurite outgrowth induced by the expression of constitutively active MKK6 was more conspicuous than that induced by the expression of constitutively active MKK3. Furthermore, treatment with SB203580, but not with PD98059 or wortmannin (data not shown), inhibited constitutively active MKK6-induced neurite outgrowth in PC12 cells.

DISCUSSION

We have recently shown that BMP-2 possesses the ability to induce the neuronal differentiation of PC12 cells (18). Unlike NGF, however, BMP-2 failed to induce the activation of either 41-/43-kDa MAP kinases (ERKs) or MEK, indicating a clear
distinction between the requirement for activation of the ERK pathway and the ability of BMP-2 to induce PC12 cell neuronal differentiation. Thus, although it has been suggested that the activation of the ERK cascade is necessary and sufficient for the induction of the differentiation of PC12 cells (27, 28), our previous findings indicated the existence of alternative signaling pathway(s) responsible for mediating the BMP-2 regulation of neuronal differentiation of these cells.

In this report, we have examined the possible involvement of other members of the MAP kinase family, JNK and p38 MAP kinase, in the BMP-2 signaling pathway that induces the neuronal differentiation of PC12 cells. These new family members of the MAP kinase are activated primarily by environmental stress and inflammatory agents (42–44), implying a role for these two MAP kinase pathways in the signaling mechanism governing the inflammatory process. However, participation of JNK and p38 MAP kinase in the regulation of a wide variety of biological processes such as cell proliferation, cell differentiation, cell survival, and immunity gene expression has also been suggested (reviewed in Refs. 25 and 26).

In PC12 cells, BMP-2 induced the selective activation of p38 MAP kinase and MAPKAP kinase-2, a downstream effector kinase of p38 MAP kinase (36). Activation of these kinases was sustained, and a considerable level of activation still remained even 4 h after stimulation of the cells (Figs. 2 and 3). Activin A, which is another member of the transforming growth factor-β superfamily that induces the neuronal differentiation of PC12 cells, also induced the activation of p38 MAP kinase and MAPKAP kinase-2. In contrast, neither ERKs nor JNK was acti-

**Fig. 6.** Expression of kinase-negative forms of MKK3/6 in PC12 cells inhibits the induction of neurite outgrowth in response to BMP-2. PC12 cells were mock-transfected (Mock) or transfected with an expression vector encoding wild-type MKK3 (WT-MKK3), the kinase-negative form of MKK3 (KN-MKK3) or the kinase-negative form of MKK6 (KN-MKK6), or a combination of KN-MKK3 and KN-MKK6. After incubating in the growth medium for 24 h and then in the serum-free medium for 12 h, the cells were treated with 30 ng/ml of BMP-2 for 60 h. A, representative images of the transfected cells as viewed by fluorescent microscopy after staining with the anti-FLAG antibody/anti-c-Myc antibody and the FITC-conjugated goat anti-mouse secondary antibody (magnification, × 140). B, cells bearing processes longer than 2 times the length of the cell body were quantified by phase-contrast microscopy examinations or by fluorescent microscopy examinations as described under “Experimental Procedures.” Each value represents the mean ± S.D. of triplicate determinations of a representative experiment. Data shown are representative of three separate experiments that gave essentially the same results.

**Fig. 7.** Expression of constitutively active forms of MKK3/6 in PC12 cells induces neurite outgrowth in the absence of BMP-2 stimulation. PC12 cells were transfected with an expression vector encoding the constitutively active form of MKK3 (CA-MKK3) or the constitutively active form of MKK6 (CA-MKK6). The cells were then treated with 0.1% Me2SO, 10 μM SB203580 (SB), or 50 μM PD98059 (PD) for 60 h. Representative images of the transfected cells as viewed by fluorescent microscopy after staining with the anti-FLAG antibody/anti-c-Myc antibody and the FITC-conjugated goat anti-mouse secondary antibody are shown (magnification, × 160). Similar results were obtained in three independent experiments.
vated in BMP-2/activin A-treated PC12 cells. All of these results suggest that the p38 MAP kinase signaling pathway, but not the JNK or ERK pathway, is involved in the induction of neuronal differentiation of PC12 cells by these agents. However, specific inhibition of the p38 MAP kinase pathway is required to clearly demonstrate a causal relationship between this pathway and the induction of PC12 cell neuronal differentiation.

To demonstrate a causal link between the activation of the p38 MAP kinase pathway and the differentiation of PC12 cells, we first employed a specific inhibitor of p38 MAP kinase, SB203580 (37). In PC12 cells, SB203580 inhibited the BMP-2/activin A-induced activation of the p38 MAP kinase pathway as measured by determining MAPKAP kinase-2 activity (Fig. 3). Importantly, this inhibition of activation of the p38 MAP kinase pathway correlated well with the ability of SB203580 to inhibit the BMP-2/activin A-induced neuronal differentiation of PC12 cells (Fig. 1). In contrast, other inhibitors such as wortmannin and PD98059 inhibited neither BMP-2-induced neurite outgrowth nor BMP-2-stimulated activity of p38 MAP kinase.

MKK3 and MKK6 are direct and specific activators of p38 MAP kinase (23–26). In order to examine more directly the role of p38 MAP kinase activity in BMP-2-induced neuronal differentiation, we tried to transiently express several mutant forms of MKK3/6 in PC12 cells. However, the transfection efficiency of CDNA into PC12 cells was around 10% in repeated experiments, which made it difficult to determine directly the effect of exogenous expression of mutant forms of MKK3/6 on p38 MAP kinase activity. For this reason, we generated PC12 cells overexpressing wild-type MKK6. Overexpression in PC12 cells of wild-type MKK6 selectively enhanced the BMP-2/activin A-induced activity of p38 MAP kinase, whose activation correlated well with the ability of these cells to induce neurite outgrowth in response to BMP-2 or activin A (Figs. 4 and 5). Finally, PC12 cells were transiently transfected with an expression vector encoding kinase-negative form of MKK3 or MKK6. Expression of the kinase-negative form of either MKK3 or MKK6 inhibited the formation of neurite outgrowth in response to BMP-2. Furthermore, co-transfection into PC12 cells of both of these kinase-negative forms blocked BMP-2-induced neurite outgrowth to a greater extent than observed in cells expressing either of them alone (Fig. 6). All of these results suggest that both MKK3 and MKK6 function as activators of p38 MAP kinase in PC12 cells and clearly support the notion that activation of the p38 MAP kinase signaling pathway is necessary for BMP-2/activin A-induced neuronal differentiation of the cells.

Expression of the constitutively active form of MKK3 or MKK6 induced neurite outgrowth without BMP-2 stimulation, which was inhibited by the treatment of the cells with SB203580 (Fig. 7). Although it has been reported that some of the biological effects of transforming growth factor-β, BMPs, and activins are mediated by the Smad pathway (20–22), our present results imply that activation of the p38 MAP kinase alone can induce the neuronal differentiation of PC12 cells. It remains to be elucidated whether or not the Smad pathway is also involved in the signaling pathway of BMP-2 to induce the neuronal differentiation of PC12 cells.

It has been shown that activation of the ERK pathway is necessary and sufficient for the neuronal differentiation of PC12 cells (27, 28, 45). However, another report demonstrated that activation of the ERK pathway alone was insufficient for growth factor receptor-mediated PC12 cell differentiation. The integration of other signals such as those of the Src/PLCγ and the ERK pathway was suggested to be required (46). Accordingly, it has been shown that NGF activates the p38 MAP kinase signaling pathway in PC12 cells to stimulate the phosphorylation of the transcription factor CREB and the activation of immediate-early genes (47). Furthermore, a requirement for p38 MAP kinase activity in NGF-induced neurite outgrowth in PC12 cells has recently been reported (48). Recently, we have also observed that the expression in PC12 cells of the kinase-negative forms of MKK3/MKK6 inhibits, although partially, the induction of neurite outgrowth in response to NGF; i.e. NGF-induced neurite outgrowths in PC12 cells expressing kinase-negative MKK3/MKK6 were apparently shorter, and, furthermore, branching and intermingling of the neurites was less conspicuous as compared with those observed in PC12 cells not expressing kinase-negative MKK3/MKK6 (data not shown). Pretreatment of PC12 cells with SB203580 (10 μM) also resulted in partial inhibition of NGF-induced neurite outgrowth. Thus, although activation of the ERK pathway alone has been suggested to be able to induce PC12 cell neuronal differentiation (27, 28, 45), it seems likely that activation of both the ERK pathway and the p38 MAP kinase pathway is required for full induction of neurite outgrowth following stimulation of PC12 cells with NGF.

p38 MAP kinase has been proposed to be involved in apoptosis (49). Accordingly, it has been shown that many apoptotic signals stimulate p38 MAP kinase activity and that p38 MAP kinase activation coincides with the induction of apoptosis in several types of cells (50–52). We have observed in repeated experiments apoptotic cell death in populations of PC12 cells transfected with the constitutively active form of MKK3 or MKK6 (data not shown). It might be that a low level of expression of the constitutively active forms of MKK3/6 (which would result in low p38 MAP kinase activity) induces the neuronal differentiation of PC12 cells, while a very high level of expression (which would result in very high p38 MAP kinase activity) leads to apoptotic cell death. In this respect, it is interesting to note that differences in the magnitude of p38 MAP kinase activation have been proposed to be a decisive factor determining the choice between cell growth and apoptosis in vascular smooth muscle cells subjected to oxidative stress (53).

In conclusion, we have demonstrated in this report that MKK3/6 and p38 MAP kinase are essential components in the BMP-2 signaling pathway leading to the induction of neuronal differentiation in PC12 cells.

Acknowledgments—We thank Drs. Roger J. Davis, Masahiko Hibi, Kenji Sugiyama, and Masatoshi Hagiwara for supplying the plasmids; Dr. Kaoru Miyamoto for activin A; and Dr. Patrick Hughes for critical reading of the manuscript.

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