Communication

Requirement of p38 Mitogen-activated Protein Kinase for Neuronal Differentiation in PC12 Cells*

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Nerve growth factor (NGF) induces sustained activation of classical MAP kinase (MAPK, also known as ERK) and neuronal differentiation in PC12 cells, whereas epidermal growth factor (EGF) induces transient activation of ERK/MAPK and stimulates proliferation of the cells. Although previous studies showed that sustained activation of ERK/MAPK is important for neuronal differentiation of the cells, a recent report revealed that inhibition of the sustained phase of ERK/MAPK activation alone does not block neurite outgrowth caused by NGF. These results suggest requirement for an additional signaling pathway(s) triggered by NGF in neuronal differentiation. Here we show that NGF induces sustained activation of p38, a subfamily member of the MAPK superfamily, and that inhibition of the p38 pathway blocks neurite outgrowth in PC12 cells. Surprisingly, expression of constitutively active MAPK/ERK kinase (MAPKK, also known as MEK) results in p38 activation as well as ERK/MAPK activation, and a p38 inhibitor blocks neurite outgrowth caused by the constitutively active MAPKK/MEK. Moreover, constitutive activation of p38 is able to induce neurite outgrowth when combined with EGF treatment. These results reveal an essential role of p38 in neuronal differentiation in PC12 cells.

The phaeochromocytoma cell line PC12 is a well studied model of actions of neurotrophic factors such as NGF1 (1, 2). NGF treatment of PC12 cells leads to their differentiation into sympathetic-like neurons characterized by neurite outgrowth. Previous studies have suggested that NGF-induced, sustained activation of the ERK/MAPK pathway (3–6) is crucial for neuronal differentiation of the cells, since blockade of the ERK/MAPK activation inhibits neurite induction, and constitutive activation of the ERK/MAPK pathway results in neurite outgrowth (7, 8). However, a recent finding that although the sustained activation of ERK/MAPK results from activation of Raf1, expression of a mutant Rap1 that blocks the sustained phase of ERK/MAPK activation does not inhibit neurite outgrowth triggered by NGF demonstrates that neurite outgrowth by NGF does not require sustained activation of ERK/MAPK (9). Moreover, there was another finding that bone morphogenetic protein-2 is able to induce neuronal differentiation of PC12 cells without marked activation of ERK/MAPK (10). Bone morphogenetic protein-2 was also reported to induce neuronal differentiation in neural crest stem cells (11). These observations suggest the existence of an additional signaling pathway(s) important for neuronal differentiation of PC12 cells. On one hand, we observed activation of p38 as well as ERK/MAPK in response to brain-derived neurotrophic factor, a member of an NGF family of neurotrophic factors, in cultured cerebellar granule cells.2 We therefore examined a possible involvement of p38 in NGF actions. We have shown that NGF treatment induces rapid and relatively long activation of p38 and that inhibition of p38 by a specific inhibitor SB203580 or by expression of dominant-negative constructs of the p38 pathway blocks neurite outgrowth in PC12 cells. Surprisingly, expression of constitutively active MAPKK/MEK resulted in p38 activation as well as ERK/MAPK activation, and treatment with the p38 inhibitor blocked neurite induction caused by the constitutively active MAPKK/MEK. Moreover, we have shown that constitutive activation of p38 is able to induce neurite outgrowth when combined with EGF treatment, which induces transient activation of ERK/MAPK and p38. These results demonstrate an essential role of sustained activation of p38 in neuronal differentiation and suggest the importance of sustained activation of either the p38 or the ERK/MAPK pathway in determining the differentiation response in PC12 cells.

EXPERIMENTAL PROCEDURES

Materials—NGF was purchased from Biomedical Technologies Inc. EGF was from Collaborative Biomedical Products. The p38 MAPK inhibitor SB203580 and the MEK inhibitor PD98059 were from Calbiochem and New England Biolabs, respectively. The phosphospecific antibody against p38 MAPK was purchased from New England Biolabs. Anti-p38 antiseraum was produced by immunizing rabbits with recombinant His-tagged p38.3 Anti-HA antibody and anti-p38 antibody were purchased from Santa Cruz Biotechnology.

Cell Culture and Preparation of Cell Extracts—Rat phaeochromocytoma PC12 cells were cultured on poly-lysine-coated plates in Dulbecco’s modified Eagle’s medium supplemented with 0.35% glucose, 10% fetal calf serum, and 5% heat-inactivated horse serum. After various treatments as indicated, the cell extracts were prepared as described previously (3).

Protein Kinase Assays and Immunoblotting—Immunoprecipitation and immune complex kinase assays were performed as described previously (12, 13). p38 MAPK and ERK/MAPK activities were assayed with His-tagged activating transcription factor 2 and myelin basic protein, respectively, as substrates. After electrophoresis, radioactivity was analyzed with an image analyzer (Bio-Rad). Immunoblotting was performed as described previously (13).

Neurite Outgrowth—PC12 cell differentiation was determined by scoring for neurite outgrowth. Cells possessing one or more neurites of a length more than 1.5-fold the diameter of the cell body were scored as positive.

Plasmids and Transfection—The expression vectors for wild type

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1 The abbreviations used are: NGF, nerve growth factor; ERK, extracellular-regulated kinase; MAPK, mitogen-activated protein kinase; MAPKK, MAPK kinase; MEK, MAPK/ERK kinase; EGF, epidermal growth factor; HA, hemagglutinin; KN, kinase-negative; GFP, green fluorescent protein.

2 T. Morooka, H. Kawasaki, Y. Gotoh, and E. Nishida, unpublished data.

3 T. Moriguchi, F. Itoh, and E. Nishida, unpublished data.

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MAPKK/MEK and a constitutively active form of MAPKK/MEK (SESE-KK) and those for wild type MKK6, HA-tagged wild type MAPK/ERK and p38 have been described previously (13, 14). A kinase-negative MKK6 (KN-MKK6) was constructed by replacing lysine 82 with alanine and inserted into pSRα vector.4 pEGFP-C1 was purchased from CLONTECH. The AGF-p38 expression vector (15) is a kind gift from Dr. Roger Davis. Transfection into PC12 cells was done by the LipofectAMINE method with the PLUS Reagent according to the manufacturer's instructions (Life Technologies, Inc.) with the use of 3 μg of total DNA/60-mm dish. Transfected cells were identified by cotransfection with pEGFP-C1, an expression vector encoding a variant of the Aequorea victoria green fluorescent protein (GFP).

**RESULTS AND DISCUSSION**

We first tested whether NGF treatment induces activation of p38 in PC12 cells. An immune complex kinase assay for p38 in extracts of PC12 cells treated with NGF revealed that p38 became activated within 5 min of the treatment, and the activity was maximal at 10 min and then gradually decreased (Fig. 1A, left). The maximal level was similar to the level of activation induced by arsenite, a well known p38-activating stimulus. A paper that reported p38 activation by NGF in PC12 cells appeared during the course of preparation of this manuscript. The NGF-induced activation of p38 was dependent on the concentration of NGF (Fig. 1A, right), and the low level of activation was sustained for more than 3 h after NGF treatment (Fig. 1A, left). The immunoblotting with an anti-phospho-p38 antibody that specifically recognizes the activated form of p38 showed similar kinetics of NGF-induced activation of p38 (Fig. 1A, anti-P-p38). The protein level of p38 did not change upon NGF treatment (Fig. 1A, anti-p38).

As the NGF-induced activation of p38 was marked, we examined the effect of inhibition of p38 on the NGF-induced neurite outgrowth in PC12 cells. Incubation of PC12 cells with SB203580, a specific inhibitor of p38 (17, 18), resulted in

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4 T. Moriguchi and E. Nishida, unpublished data.

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**FIG. 1.** NGF induces p38 activation as well as ERK/ MAPK activation. A, PC12 cells were treated with 100 ng/ml NGF or 50 μM arsenite for the indicated times (left) or with the indicated concentrations of NGF for 10 min (right), and the cell extracts were subjected to the immune complex kinase assay for p38 using activating transcription factor 2, ATF2, as a substrate (upper). The same cell extracts were subjected to immunoblotting with anti-phospho-p38 (middle) or anti-p38 antibodies (bottom). B, cells were pretreated with or without a p38 inhibitor SB203580 at 10 μM (lower or upper, respectively) for 30 min prior to NGF treatment as indicated, and the extracts were subjected to immunoblotting with anti-ERK/MAPK antibody. The electrophoretically retarded bands represent active forms, i.e. phosphorylated forms of ERK/MAPK (ERK1 and ERK2, arrowheads) against inactive forms (arrows).
marked inhibition of the NGF-induced neurite outgrowth; the effect being dependent on the concentration of SB203580 (Fig. 2, A and B). The SB203580 at 10 μM was as effective as 30 μM PD98059, a specific inhibitor of MAPKK/MEK (8), in blocking neurite outgrowth (Fig. 2, A and B). Although the NGF-induced activation of ERK/MAPK was inhibited by 30 μM PD98059 by more than 85% (data not shown), it was not inhibited at all by 10 μM SB203580 (Fig. 1B). Therefore, the sustained activation of ERK/MAPK alone is not sufficient for neurite induction, and the activated p38 activity is required for the NGF-induced neurite outgrowth. To confirm this, we expressed dominant-negative constructs of the p38 pathway in PC12 cells. Expression of a kinase-dead form of MKK6 (MKK6 is a direct and specific activator of p38) significantly blocked neurite outgrowth induced by NGF (Fig. 2, C and D, KN-MKK6). Furthermore, expression of a nonactivatable form of p38 (AGF-p38, in which threonine and tyrosine residues in the activation phosphorylation site were replaced by alanine and phenylalanine, respectively), but not that of wild type p38, resulted in marked inhibition of the neurite outgrowth (Fig. 2, C and D, AGF-p38, WT-p38). These results clearly indicate that p38 activation is necessary for NGF-induced neurite outgrowth in PC12 cells.

The previous finding that expression of a constitutively active mutant of MAPKK/MEK, a direct activator of ERK/MAPK, is capable of inducing neurite outgrowth in PC12 cells in the absence of NGF (7) (see Fig. 3A) seemed to apparently contradict the above results. Surprisingly, however, we have found that expression of the constitutively active MAPKK/MEK (=SESE-KK) induces constitutive activation of p38, although the level of the activation is lower than the maximal level of p38 activation induced by NGF treatment (Fig. 3B). This acti-
vation of p38 was also demonstrated by immunofluorescent cell staining with anti-phospho-p38 antibody of SESE-KK-expressing cells (Fig. 3C). The activation of p38 was dependent on the activated kinase activity of MAPKK/MEK, as expression of wild type MAPKK/MEK did not induce the activation of p38 (Fig. 3B, W7-KK). Although the mechanism by which MAPKK/MEK activation results in p38 activation remains to be revealed, it may be an indirect effect, because the activated forms of MAPKK/MEK, including dually phosphorylated MAPKK/MEK and SESE-MAPKK/MEK purified from the expressed cells, did not directly activate p38 in vitro (data not shown). Remarkably, treatment with a p38 inhibitor SB203580 at 10 μM blocked the constitutively active MAPKK/MEK (SESE-KK)-induced neurite outgrowth in PC12 cells (Fig. 3A). In these cells, ERK/MAPK was strongly and constitutively activated (Fig. 3B), indicating again that sustained activation of ERK/MAPK alone, even if strongly activated, is not sufficient for neurite induction and that p38 activation is necessary.

The PC12 cell line has been extensively used as an experimental system to study the specificity of the receptor tyrosine kinase signaling; although NGF induces neuronal differentiation, EGF does not (2). If EGF treatment could not activate p38 in PC12 cells, we could hypothesize that p38 activation would determine the differentiation. However, we found that EGF treatment of PC12 cells induces strong activation of p38 (Fig. 4A and B). This is consistent with the recent report showing EGF-induced activation of p38 in NIH-3T3 cells (16). Our detailed analysis, however, revealed a marked difference in the kinetics of p38 activation between EGF and NGF treatments. The EGF-induced activation of p38 was more transient than the NGF-induced activation. The immune complex kinase assay showed that the EGF-induced activation declined to near basal levels by 30 min, whereas the NGF-induced activation remained higher than the basal level even at 120 min (Fig. 4A). In the indirect immunofluorescent staining with anti-phospho-p38 antibody, the NGF-induced activation of p38 was clearly observed in PC12 cells at 60 min after stimulation (Fig. 4B, NGF 60′). Thus, it may be the duration of p38 activation that is important for determining the cellular response. Then, to examine whether sustained activation of p38 alone is able to induce neurite outgrowth in PC12 cells, we overexpressed both p38 and MKK6 in PC12 cells. As MKK6 alone is a relatively high basal activity (12), overexpression of both MKK6 and p38 leads to strong and constitutive activation of p38, the level comparable with the maximal level of activation of endogenous p38 induced by NGF (data not shown). However, this overexpression did not induce neurite outgrowth in the cells, although it did induce some morphological changes (Fig. 4C). Therefore, constitutive activation of p38 alone is not sufficient for neurite induction. Importantly, when combined with EGF treatment, the constitutive activation of p38 was able to induce neurite outgrowth (Fig. 4C), which was blocked by the p38 inhibitor SB203580 (Fig. 4C). The constitutive activation of p38 did not change the transient nature of the EGFinduced activation of ERK/MAPK (data not shown). We further found that arsenite treatment plus EGF treatment led to neurite induction in PC12 cells (Fig. 4D), which was also blocked by SB203580 (data not shown). The EGF treatment induces transient activation of both ERK/MAPK (3, 4, 6) and p38 (see Fig. 4A), and the arsenite treatment is shown to induce relatively sustained activation of p38 (19). Therefore, sustained activation of p38 in combination with transient activation of ERK/MAPK may be sufficient for neurite induction in PC12 cells.

An attractive model has been proposed previously that differentiation or proliferation responses are determined by the duration of ERK/MAPK activation (2), as there was considerable evidence that neuronal differentiation of PC12 cells correlates with sustained activation of ERK/MAPK (6, 20–22). This model, however, is apparently inconsistent with the observation that blockade of the sustained phase of ERK/MAPK activation does not inhibit NGF-induced neurite outgrowth (9, 23, 24). Our results here demonstrate that NGF induces longer activation of p38 than does EGF, which is required for neurite induction, and that sustained activation of p38 in combination with EGF treatment is able to induce neurite outgrowth. From these results the above inconsistency would be dissolved and a modified model could be formed; sustained activation of either of the two signaling pathways, the ERK/MAPK or the p38 pathway, combined with transient activation of the other or both, is critical for neuronal differentiation in PC12 cells. Thus, control of the duration of the both pathways may underlie the mechanism that determines the specificity of the receptor tyrosine kinase signaling.

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