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SB203580 Promote EGF-Stimulated Early Morphological Differentiation in PC12 Cell Through Activating ERK Pathway

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Abstract MAP kinases have important role in PC12 cell differentiation, since the activities of both extracellular regulated protein kinase (ERK) and p38 have been indicated as necessary signal for PC12 cell differentiation. Epidermal growth factor (EGF) and NGF both activate ERK and p38 in PC12 cells, but only NGF trigger differentiation. It has been proposed that the duration of ERK activation determines the switch from proliferation to differentiation, since EGF causes more transient activation of ERK than NGF in PC12 cells. Here we report that treatment of PC12 cells with EGF in the presence of SB203580, a widely used p38 inhibitor, caused differentiation. The pro-differentiation effect of SB203580 in EGF-treated PC12 cells was found to be independent of its function of p38 inhibition but was through an effect on the ERK pathway that has been recently reported (Kalmes et al. [1999] FEBS Lett. 444: 71–74; Hall-Jackson et al. [1999] Onc. 18: 2047–2054). We found that SB203580 by itself did not affect the activity of ERK1/2 but significantly extended EGF-induced ERK activation in PC12 cells, which resulted in early morphological differentiation. Our data indicated that although both ERK and p38 are required for PC12 cell differentiation, activation of p38 is not required when ERK is superactivated. Our data provided further evidence for the threshold theory that differentiation is determined by the duration of ERK activation. J. Cell. Biochem. 83: 585–596, 2001.

Key words: MAP kinase; SB203580; differentiation; EGF; NGF

MAP kinases have received much attention in the study of signal pathways that control neuronal differentiation. Constitutive activation of MEK1, a direct activator of ERK1/2, has been proposed to be both necessary and sufficient for PC12 cell differentiation [Cowley et al., 1994; Dudley et al., 1995]. There is evidence suggesting that the ERK1/2 is the major kinase responsible for the N-terminal phosphorylation of c-Jun in PC12 cells [Pulverer et al., 1991; Leppa et al., 1998], although JNK has been proposed to be the activator of c-Jun in other cell types or in in vitro kinase assays [Minden et al., 1994]. Phosphorylation of c-Jun is believed to be the mechanism by which ERK triggers PC12 cell differentiation [Leppa et al., 1998]. Another MAP kinase, p38, has also been suggested to be both required [Morooka and Nishida, 1998; Iwasaki et al., 1999] for neuronal differentiation of PC12 cells.

PC12 cells have been shown to express receptors for and to respond to both NGF and EGF [Chandler and Herschman, 1980; Huff et al., 1981]. Both NGF and EGF stimulation can lead to ERK and p38 activation as well as c-Jun expression in PC12 cells [Greenberg et al., 1985; Wu et al., 1989; Wixler et al., 1996; Morooka and Nishida, 1998]. However, NGF causes PC12 cells to stop dividing and to differentiate, whereas EGF promotes PC12

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cells to continue to proliferate without evident morphological alteration [Huff et al., 1981; Maher, 1988]. It has been proposed that although signals from the two receptors may be involved in similar or partly overlapping pathways, temporal and quantitative differences in intracellular biochemical reactions resulting from activation of two receptors lead to distinct physiological and morphological changes.

MAP kinase inhibitors have proved to be very useful tools in exploring functions of individual MAP kinase pathway. For example, SB203580 [4-(4-fluorophenyl)-2-(4-methylsulfinyl-phenyl) -5-(4-pyridyl)1H-imidazole] has been extensively used as a p38 inhibitor both in vitro and in vivo. However, recent studies have found that SB203580 over 5 μM can activate Raf-1independently from its inhibitory effect on p38. Although Raf-1 activity was increased, its downstream MAP kinase, ERK1/2, were not activated by SB203580 treatment alone in intact cells [Hall-Jackson et al., 1999; Kalmes et al., 1999]. In combination with EGF, SB203580 can enhance ERK1/2 activation but the physiological consequence of the SB203580 effect on the ERK pathway has not been revealed [Hall-Jackson et al., 1999].

Here we report that SB203580 promotes early morphological differentiation in EGF-treated PC12 cells. The promotion of differentiation by SB203580 was not due to its inhibitory effect on p38 but occurred through its enhancing effect on the level and duration of ERK activation in EGF-treated PC12 cells. Our data provided evidence supporting the proposition that the temporal control of ERK1/2 activation determines the different biological effect of NGF and EGF in PC12 cells and also suggested that superactivation of ERK pathway can override the negative effect of p38 inhibition in early stage of neuronal differentiation.

MATERIALS AND METHODS

Reagents

SB203580 and U0126 were purchased from Calbiochem (San Diego, CA). EGF, FGF-2, activin A, and NGF were purchased from R&D Systems (Minneapolis, MN). Polyclonal antibodies to TFIIB and to MMP-3 were purchased from Santa Cruz Biotechnology, Inc., (Santa Cruz, CA) and primary antibodies for immunoassay including polyclonal antibodies to phospho p38, phospho ERK/12, phospho MEK1, phospho JNK1/2, c-Jun, phospho c-Jun (73), and phospho c-Jun (63) were purchased from New England BioLabs, Inc., (Beverly, MA). Rabbit anti-p38 and anti-MAPKAPK-2 polyclonal antibodies were raised using recombinant human p38 and MAPKAPK-2. HSP-27 was expressed as a his-tagged recombinant protein expressed and purified as described previously [New et al., 1998]. Protein A and protein G beads were purchased from Santa Cruz and Calbiochem.

Cell Culture

The PC12 cell line, purchased from American Type Culture Collection (ATCC, Manassas VA), was maintained in Dulbecco's modified Eagle's medium plus 10% fetal bovine serum and 5% heat-inactivated horse serum at 37°C in a humidified 5% CO_2 atmosphere. For differentiation assays, cells were plated at 30–40% coverage under the above conditions 24 h before induction. For biochemical analysis, cells were seeded in 6-well plates at 80–90% confluence and incubated under the same culture conditions overnight. The next morning, the medium was changed to a low serum formulation (Dulbecco's modified Eagle's medium plus 1% horse serum) for 5 h before treatment. Kinase inhibitors were added 30 min before growth factors. Image of cell morphological states of differentiation was taken by SPORT digital camera (Diagnostic Instruments Inc., Detroit, MI) which connected to an Axiovert 100 TV microscope (Carl Zeiss Inc., Denver, CO).

Infection of Adenoviral Expression Vectors in PC12 Cells

The construction of dominant negative p38α [p38(AF)] and the green fluorescent protein (GFP) adenovirus vectors was previously described elsewhere [Huang et al., 1997; Kato et al., 1998]. To analyze the effect of the dominant negative p38 on differentiation and on the activities of endogenous p38 or ERK1/2 stimulated by different reagents, PC12 cells cultured in normal serum medium were infected with p38(AF) or control GFP virus at a titer of 10 plague-forming units/cell. After 16 h of infection, cells were washed with 1 × PBS and continually cultured in fresh medium. Various reagents were added at this point. Total proteins from each treatment were extracted at different times as indicated in Figure 3. States

of differentiation were photographed days after the treatments.

Western Blot Analysis

Cells in 6-well plates under different treatments were directly lysed by adding one volume of 1 × lysing buffer (New England BioLabs, Inc.) and one volume of SDS loading buffer. The whole-cell lysate was boiled for 5 min and spun in a bench top microcentrifuge at maximum speed for 10 min. Protein samples were separated on 12% SDS–PAGE and electroblotted onto Hybond P membrane (Amersham Pharmacia Biotech, Piscataway, NJ). After blocking and incubation with relevant primary antibodies, the membranes were incubated with HRP-conjugated secondary antibody (Amersham Pharmacia Biotech) and developed with a chemiluminescent system (ECL+ plus, Amersham Pharmacia Biotech). In some cases, the membrane was stripped with stripping buffer (100mM 2-Mercaptoethanol, 2% SDS and 63 mM Tris, pH 7.0) at 50°C for 30 min and reused to detect other protein by Western blotting.

Immunoprecipitation and In Vitro Kinase Assay

Cells (in one well of 6-well plate) from different treatments were lysed in 1 × lysing buffer and incubated with MAPKAPK-2 polyclonal antiserum and a mixture of protein A and Protein G beads at 4°C for 4 h. After 3 times of wash with 1 × lysing buffer and one times of kinase buffer, half volume of the beads were transferred to a kinase reaction tube containing 10 μg of HSP-27 and 10 μCi of γ-^{32}p ATP and were incubated on a rotating shaker at 37°C for 1 h. Each of total kinase reactions was separated on SDS–PAGE.

Quantification of Neurite Outgrowth

In various treatments, at each point of time as indicated in the figures, the percentage of cells with one or more neurites that were equal to or longer than the cell diameter was counted for 100 cells under a microscope. The data represent mean values±SE of three separate experiments.

RESULTS

Induction of PC12 Cell Differentiation by EGF in the Presence of SB203580

In order to compare the roles of the p38 and ERK pathways in regulating neuronal differentiation of PC12 cells, we investigated the effect of SB203580, a p38 inhibitor, on NGF- or EGF-treated cells. As shown in Figure 1, NGF treatment of proliferating PC12 cells caused neuronal differentiation, whereas treatment with EGF did not influence PC12 cell morphology (Fig. 1, panel b and c, respectively). In contrast to a reported experiment [Morooka and Nishida, 1998], treatment of cells with SB203580 did not inhibit NGF-induced differentiation (Fig. 1, panel d). Interestingly, we observed that addition of SB203580 (10–20 μM) to EGF-treated PC12 cells even led the cell to an early morphological differentiation (Fig. 1, panel e). SB203580 alone can not lead the cell to the same state (panel f). We quantitatively compared the responses of PC12 cells to the treatment of EGF, NGF and SB203580 alone or EGF and NGF in combination with SB203580. This was achieved by calculating the percentage of the cells with neurites at different time points (Fig. 2A). Treatment of SB203580 alone did not cause any significant morphological change on PC12 cells. EGF alone only had a slight enhancement of neurite outgrowth at a later time point. EGF plus SB203580 promoted neurite outgrowth to a similar level as NGF treatment within 72 h. NGF combined with SB203580 showed an earlier onset of neurite outgrowth than that treated by NGF alone.

Since the expression of MMP-3 (transin/stromelysin) has been implicated in various extracellular matrix remodeling and used as a biochemical indicator of early events in PC12 cell differentiation [Machida et al., 1991; Fillmore et al., 1992], we further monitored induction of MMP-3 from cells under different treatments by a Western blot (Fig. 2B). The profile of MMP-3 induction by EGF, SB203580 plus EGF and NGF basically matches the morphological responses of the cell for the corresponding treatments. These data suggest either that p38 activity is an inhibitory signal for differentiation in EGF-treated PC12 cells or that a function of SB203580 other than inhibiting p38 promotes EGF-treated PC12 cell to an early differentiation state.

Dominant Negative p38 Inhibits Endogenous p38 Activity, but Fails to Promote EGF-Induced PC12 Cell Differentiation

To verify the role of p38 in PC12 cell differentiation, we infected cells with recombinant adenovirus that express dominant negative p38 [p38(AF)] to inhibit endogenous p38

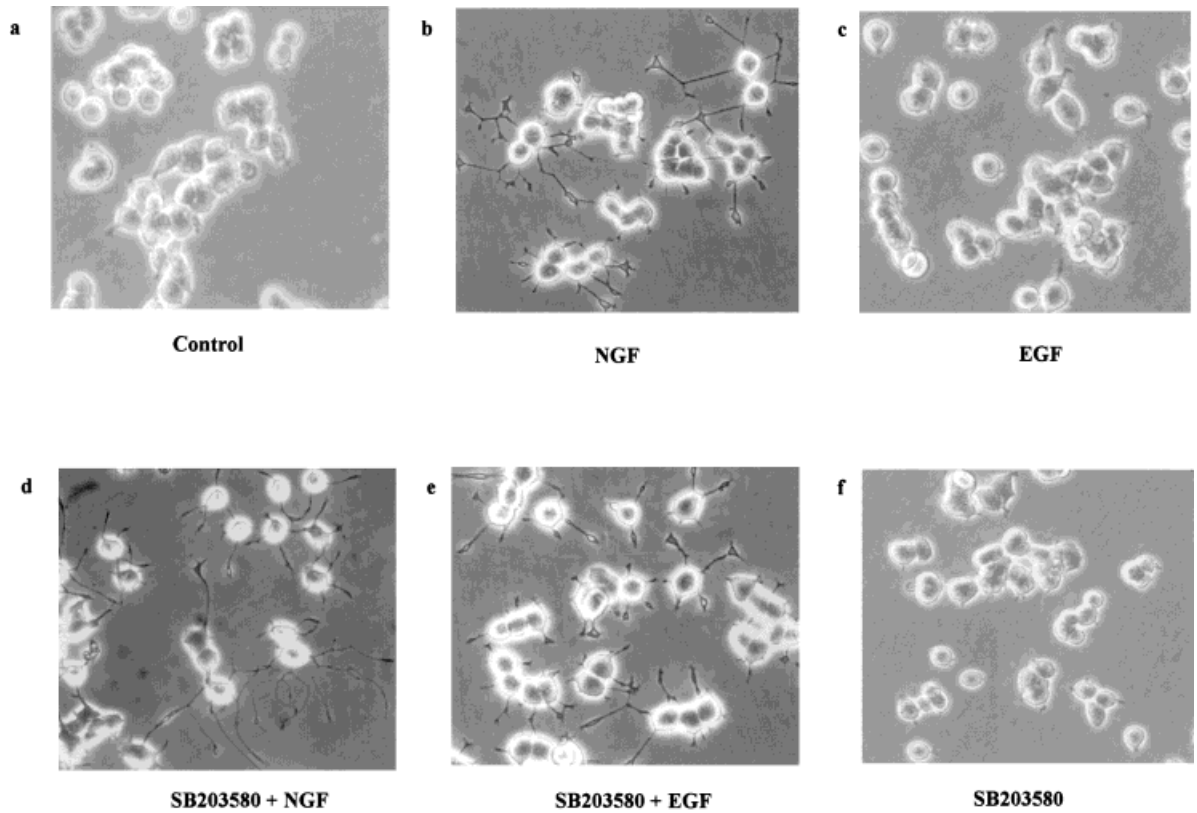

Fig. 1. The effects of SB203580 on the morphological neurite outgrowth in PC12 cells induced by NGF and EGF. PC12 cells grown in normal growth medium were used as the control **(a)** or treated with NGF (100 ng/ml) **(b)**; EGF (200 ng/ml) **(c)**; SB203580 (10μM) + NGF (100 ng/ml) **(d)**; SB203580 (10 μM) + EGF (200 ng/ml) **(e)**; and SB203580(10 μM) **(f)**. SB203580 was added 30 min before NGF or EGF. The phase-contrast photographs were taken 3 days after treatment.

activity. The infection efficiency of adenovirus in PC12 cells approached 100% as accessed by a control adenovirus GFP (green fluorescent protein) vector infection (data not shown). As shown in Figure 3A, unlike EGF + SB203580-treatment (see Fig. 1, panel e), inhibition of p38 by p38(AF) in EGF-treated PC12 cells did not promote differentiation (panel b). Instead, the expression of dominant negative p38 showed an inhibitory effect on NGF induced differentiation (panel c), which indicated that p38 activation is required for NGF-induced neuronal differentiation. GFP expression from the control vector did not affect NGF-induced differentiation (panel d) as compared to noninfected cells (panel a). As shown in Figure 3B, p38(AF) infection significantly inhibited p38 activation induced by NGF or EGF treatment, while control AdGFP had no effect. The inhibition of p38 by p38(AF) was specific, since p38(AF) infection had no influence on ERK1/2 activation. As an independent assay to determine whether p38(AF) infection has a similar range of p38 inactivation as the SB203580 treatment to EGF stimulation, we performed an p38 downstream kinase assay in which endogenous MAPKAPK-2 was immunoprecipitated from the cell and used to phosphorylate HSP-27. The result shown in Figure 3C indicated that p38 activation by EGF can be suppressed by either SB203580 or p38 (AF) infection. Therefore, the promoting effect of SB203580 on cell differentiation in EGF-treated PC12 cells is unlikely to be through inhibition of p38.

Since p38(AF) expression plus SB203580 treatment caused rapid cell death (data not shown), we were unable to test whether p38(AF) had affected SB203580+EGF-induced cell differentiation. Nevertheless, our data imply that SB203580 has a dual effect on PC12 cell differentiation: one is opposing differentiation by inhibiting p38 and the other is promoting differentiation by an unknown mechanism. The pro-differentiation role of SB203580 apparently overcame its anti-differentiation effect in our system. It was noted that SB203580 itself

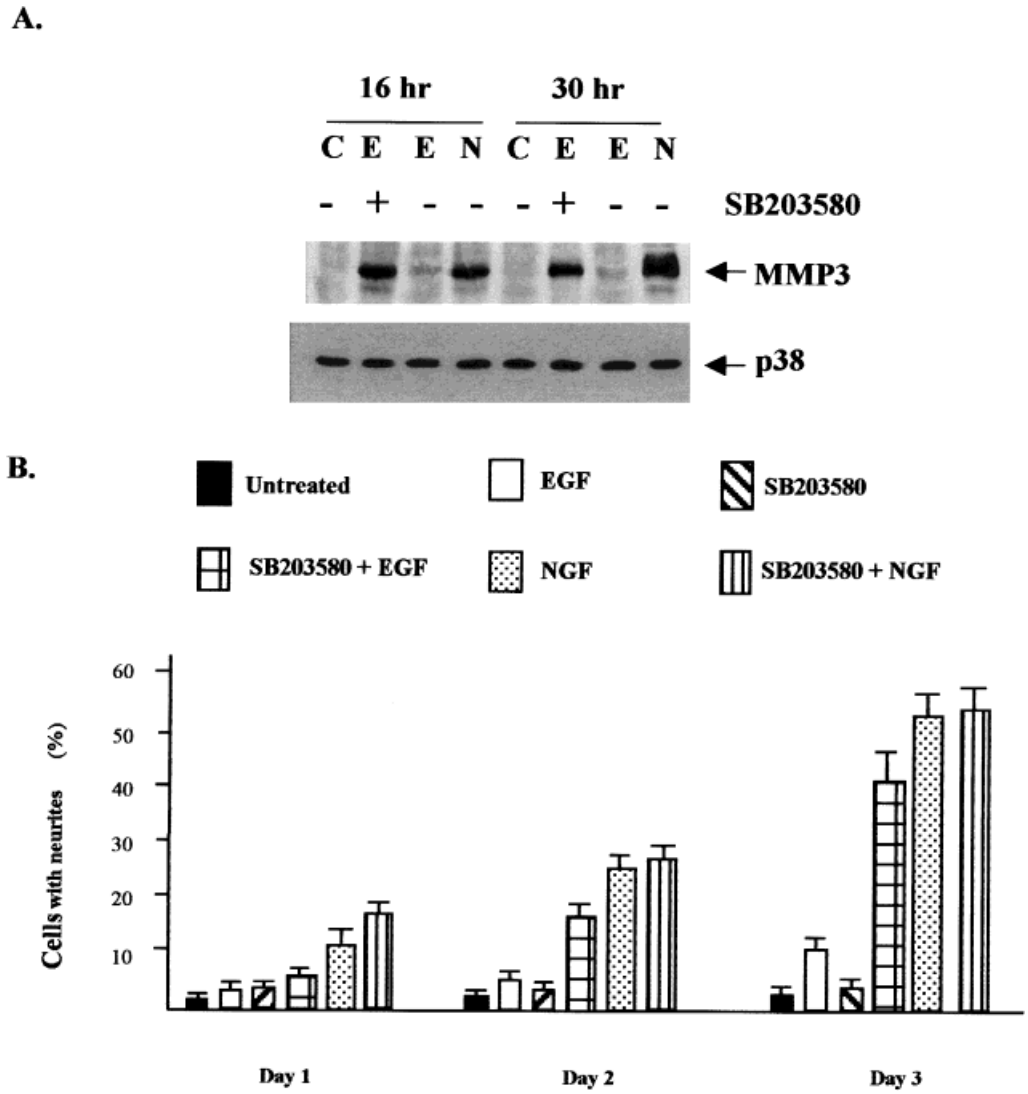

Fig. 2. Neurite outgrowth and MMP-3 expression in PC12 cells. **A:** Neurites from PC12 cells under the different treatments as indicated were measured at day 1, day 2, and day 3, respectively (see Materials and Methods). Cells with neurites equal to, or longer than, the cell diameter were counted as positive. The percentage value is quantified by microscopy from a random selection of 100 cells from three different areas and represents the mean±SD. **B:** Western blot to detect MMP-3 induction by different treatments in PC12 cells. C, for control; E, for EGF (200 ng/ml); N, for NGF (100 ng/ml).

had no effect on PC12 cell differentiation (Fig. 1, panel f) but concordance with other stimuli, such as EGF, is required for SB203580 to exert its pro-differentiation effect.

SB203580 Enhances (Prolongs) NGF- and EGF-Induced ERK Activation in PC12 Cells

An important difference between the responses of PC12 cells to NGF and EGF has been identified by previous studies, in that ERK activation persisted for several hours following NGF stimulation but was short-lived after EGF stimulation [Lai et al., 1990; Traverse et al., 1992; Traverse et al., 1994]. This result was confirmed in our experimental system (Fig. 4A). Since MEK1/2 has been indicated as potential activators of ERK1/2 in vivo [Derijard et al., 1995; Butch and Guan, 1996], we also investigated MEK1/2 activation in the experiment. Activation of ERK1/2 was correlated with that of MEK1/2, suggesting its prolonged activation profile after NGF stimulation was determined at or upstream of MEK1/2. To determine the effect of SB203580 on EGF-induced ERK1/2 activity in PC12 cells, we compared phosphorylation of MEK1/2 and ERK1/2 treated by EGF and by EGF plus SB203580. As shown in Figure 4B, addition of SB203580 to EGF

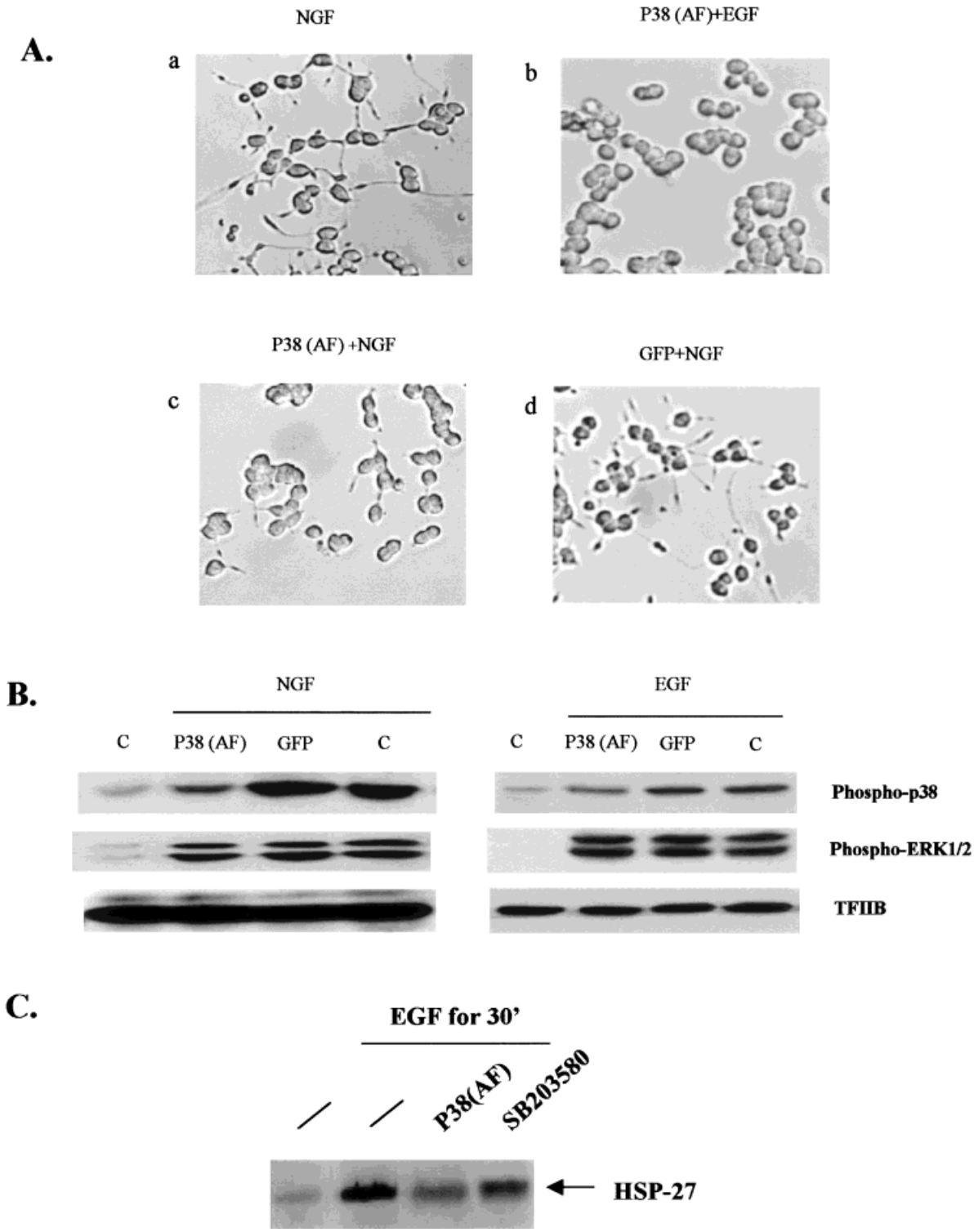

Fig. 3. The effects of dominant negative p38 on endogenous p38 and ERK activities and on PC12 cell differentiation. **A:** NGF- or EGF- induced neurite outgrowth from the cells after 2 days of infection with or without recombinant adenovirus expressing dominant negative p38, p38 (AF); or GFP as indicated. **B:** PC12 cells were infected with or without recombinant adenovirus as indicated. Total proteins were subjected to Western analysis to compare the endogenous ERK and p38 activities stimulated by NGF (100 ng/ml) or EGF (200 ng/ml) for 30 min. **C:** Activities of endogenous MAPKAPK-2 from PC12 cells were assessed by an in vitro kinase assay. HSP-27 was used as the phosphorylation substrate of MAPKAPK-2. As indicated for different treatments, PC12 cells were either infected or uninfected with p38 (AF) adeno-vector. Sixteen hours after infection, cells were treated with EGF (200 ng/ml) for 30 min. One part of uninfected cells was also treated with the same amount of EGF, but with SB203580 (10 μM) pretreated for 30 min. Total endogenous MAPKAPK-2 was immunoprecipitated as described in Materials and Methods.

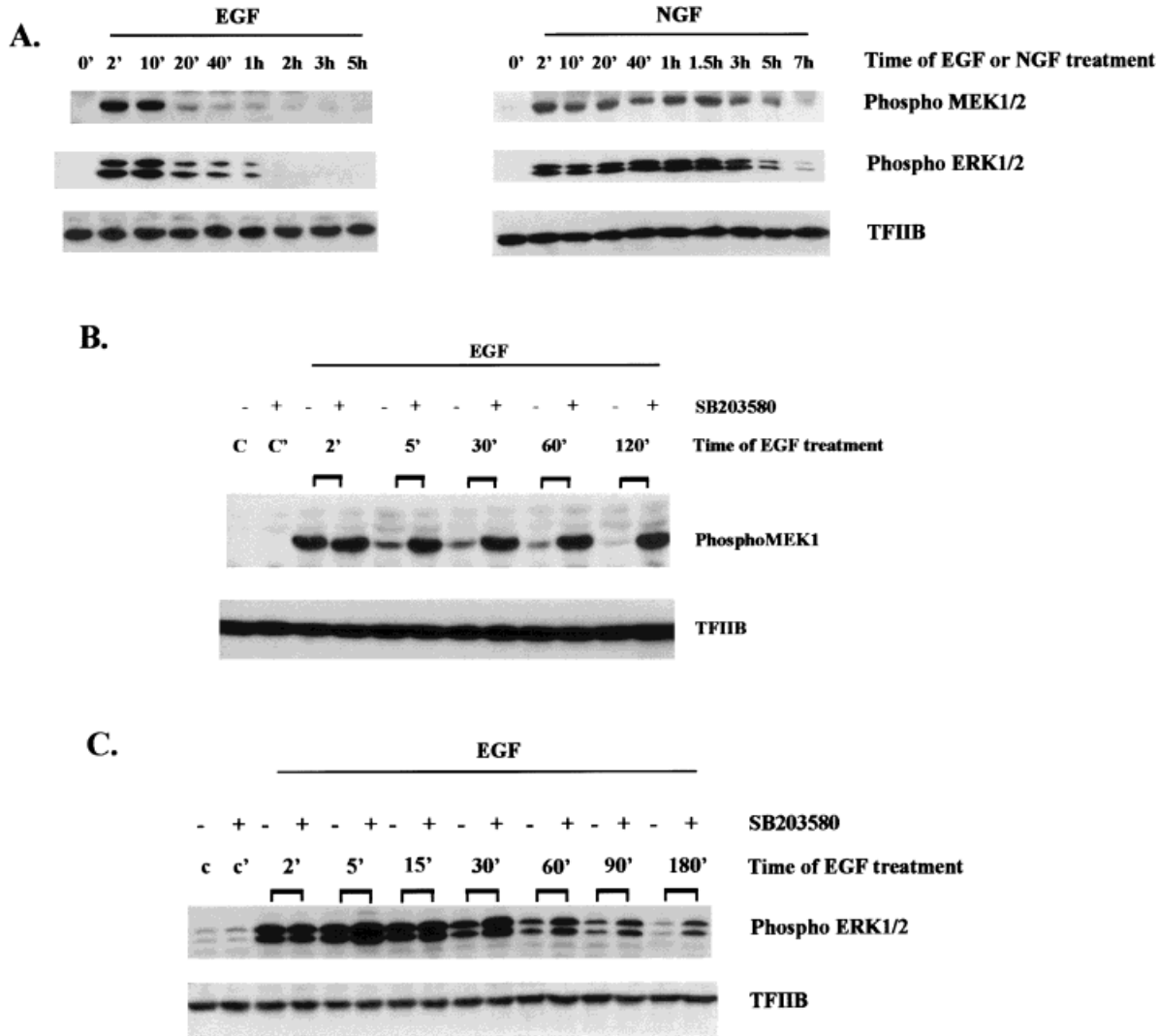

Fig. 4. Comparison of NGF- and EGF-stimulated activation of ERK1/2 and MEK1/2 in PC12 cells. **A:** Time course of activated phospho-MEK1/2 (top) and phospho-ERK1/2 (middle) after NGF or EGF treatment were analyzed by Western blot. **B:** Time course of MEK1/2 activation by EGF with or without SB203580. **C:** Time course of ERK1/2 activation by EGF with or without SB203580. In the case of a combination of SB203580 and EGF treatment, SB203580 was added 30 min before EGF. The blots were stripped after the first Western and reblotted with anti-TFIIB to judge equal amounts of protein loading.

treatment significantly prolonged activation of MEK1/2 by maintaining MEK1/2 activity at its maximum level for at least 2 h. Correspondingly, SB203580 significantly enhanced EGF-induced ERK1/2 activity as compared to EGF-treated controls ment at each time point (Fig. 4C). However, SB203580 alone seemed not to affect activation of MEK1/2 or ERK1/2 in comparison to the control (cells treated with neither SB203580 nor EGF). In a parallel experiment using NGF, it was observed that although NGF alone caused continuous activation of MEK1/2 and ERK1/2 over several hours, addition of SB203580 further increased NGF-induced phospho-MEK1/2 and phospho-ERK1/2 signals at each time point (data not shown).

SB203580 Enhances EGF-Induced c-Jun Expression Probably Through N-Terminal Phosphorylation of c-Jun

Since c-Jun is believed to be a target of ERK in PC12 cells and N-terminal phosphorylation of c-Jun has been indicated as a trigger for PC12 cell differentiation [Pulverer et al., 1991; Leppa et al., 1998], we wanted to determine whether SB203580 enhanced EGF-induced ERK activity would affects the status of c-Jun in the cell. We first compared the expression of c-Jun in NGF

and EGF treated cells. It was evident that although the maximum intensities of c-Jun signals induced by the two factors were about the same, the signal induced by EGF appeared to be much more transient than that induced by NGF (Fig. 5A). It has been known that after its induction by various stimuli, c-Jun is rapidly degraded by ubiquitination and that the N-terminal phosphorylation of c-Jun inhibits this process [Musti et al., 1997]. Thus, we subsequently examined the effect of SB203580 on EGF-induced c-Jun expression and phosphorylation. SB203580 at 2 μM or more caused enhancement of c-Jun expression in EGF-treated cells (Fig. 5B). As shown in Figure 5C, more stabilized c-Jun was detected in SB203580 treated samples than in controls treated with only EGF at each time point after protein synthesis was stopped with cycloheximide. Figure 5D shows that SB203580 concomitantly enhanced phosphorylation of EGF-induced c-Jun, when a specific serine 73 (of c-Jun) antibody was used in a similar Western blot as in Figure 5C. Therefore, SB203580 is likely, via upregulation of ERK activity, to enhance c-Jun phosphorylation and thereby increase c-Jun stability and its activity in the cell thus promoting differentiation.

ERK Activity was Required in NGF- and EGF+SB203580-Induced PC12 Cell Differentiation

To re-evaluate the role of ERK activity in NGF- and EGF+SB203580- induced PC12 cell differentiation, we used U0126 to specifically block MEK1/2 [Favata et al., 1998] in an experiment similar to Figure 1. U0126 almost completely blocked NGF- as well as EGF+203580-induced PC12 cell differentiation (Fig. 6, panel c and f). In contrast to the effects of SB203580 in EGF-treated PC12 cells, U0126 significantly inhibited EGF-induced ERK1/2 activation and c-Jun expression but without influence of p38 or JNK activity (Fig. 7). Thus, both NGF- and EGF+SB203580-induced PC12 cell differentiation require activation of the ERK pathway.

DISCUSSION

In this study, we report a pro-differentiation effect of SB203580 on EGF treated PC12 cells. This effect was independent of inhibition of p38, but due to enhancing (prolonging) EGF-induced MEK/ERK activities. Our data indicated that although both ERK and p38 are involved in PC12 cell differentiation, the ERK pathway had a predominant role over p38 since ERK superactivation can overcome the inhibitory effect of p38 inhibition on differentiation.

Specific and Non-Specific Effect of SB203580

SB203580 has been widely used as a p38 specific inhibitor and is a very useful tool in study of the function of p38. It has been reported that at high concentration, SB203580 had inhibitory effect on JNK [Chen et al., 1998]. This observation has been taken into a consideration by many investigators in interpreting the experimental results. The effect of SB203580 on Raf-1 was initially identified by in vitro kinase assay that Raf-1 can be inhibited by SB203580 at a high dose. Howevere, Kalmes et al. and Hall-Jackson et al. recently found that SB203580 could actually cause Raf-1 activation in cells by an unknown mechanism [Hall-Jackson et al., 1999; Kalmes et al., 1999]. The concentration of SB203580 required for enhancing ERK activation is comparable to the dose required for p38 inhibition in our experimental system. Further SB203580 at 2 μM can enhance ERK activation if an ERK stimulus is applied simultaneously. Thus, the ERK activity should be examined when SB203580 is used. It is also notable that a combination of p38(AF) and SB203580 was toxic to the cell, suggesting either that the profound inhibition of p38 by both agents is toxic or that SB203580 may have further undiscovered effect.

Role of p38 in PC12 Cell Differentiation

Previous studies reported p38 activation to be required for NGF-, morphogenic protein-2 (BMP-2)- or activin A-induced PC12 cell differentiation [Morooka and Nishida, 1998; Iwasaki et al., 1999]. In these experiments, SB203580 blocked PC12 differentiation. Consistence with their conclusions, our data using dominant negative p38 showed that p38 activation does indeed play a positive role in PC12 cell differentiation. However, SB203580 produced different effects in our experimental system. Similar to what we observed, fibroblast growth factor-2 (FGF-2)-induced PC12 cell differentiation was associated with both ERK and p38 activation, and SB203580 affected cell growth in this case but not the differentiation [Maher, 1999]. We do not know the cause of the

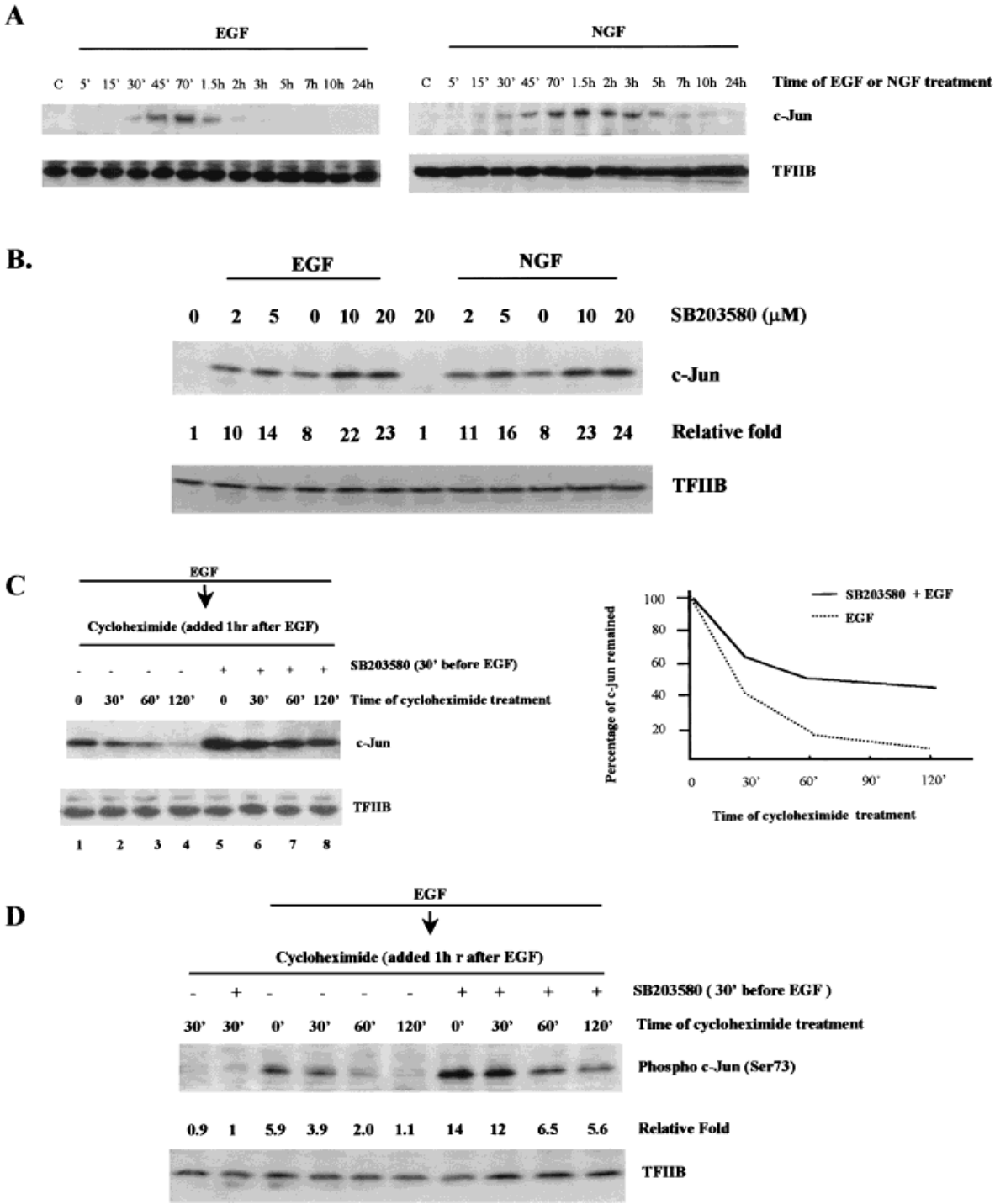

Fig. 5. The effect of SB203580 on EGF- or NGF-stimulated c-Jun expression in PC12 cells. **A:** PC12 cells in each six well plates were treated with EGF or NGF for different times as indicated and Western blotted with anti-c-Jun. **B:** The dose effect of SB203580 on EGF- and NGF-stimulated c-Jun. The relative fold of enhancement of c-Jun in each lane was based on a densitomic scan of band intensities. **C:** The effect of SB203580 on EGF-induced c-Jun protein stability. Global protein synthesis was inhibited by adding cycloheximide (50 μM) 1 h after the addition of EGF. c-Jun protein stabilities in different treatments were monitored by Western blot and quantitated as percentage remaining of c-Jun in comparison with the amount of c-Jun protein at time 0. **D:** The amounts of phosphorylated c-Jun (Ser73) in cells treated as in (C) were analyzed by another Western blot using anti-phospho-c-Jun antibody.

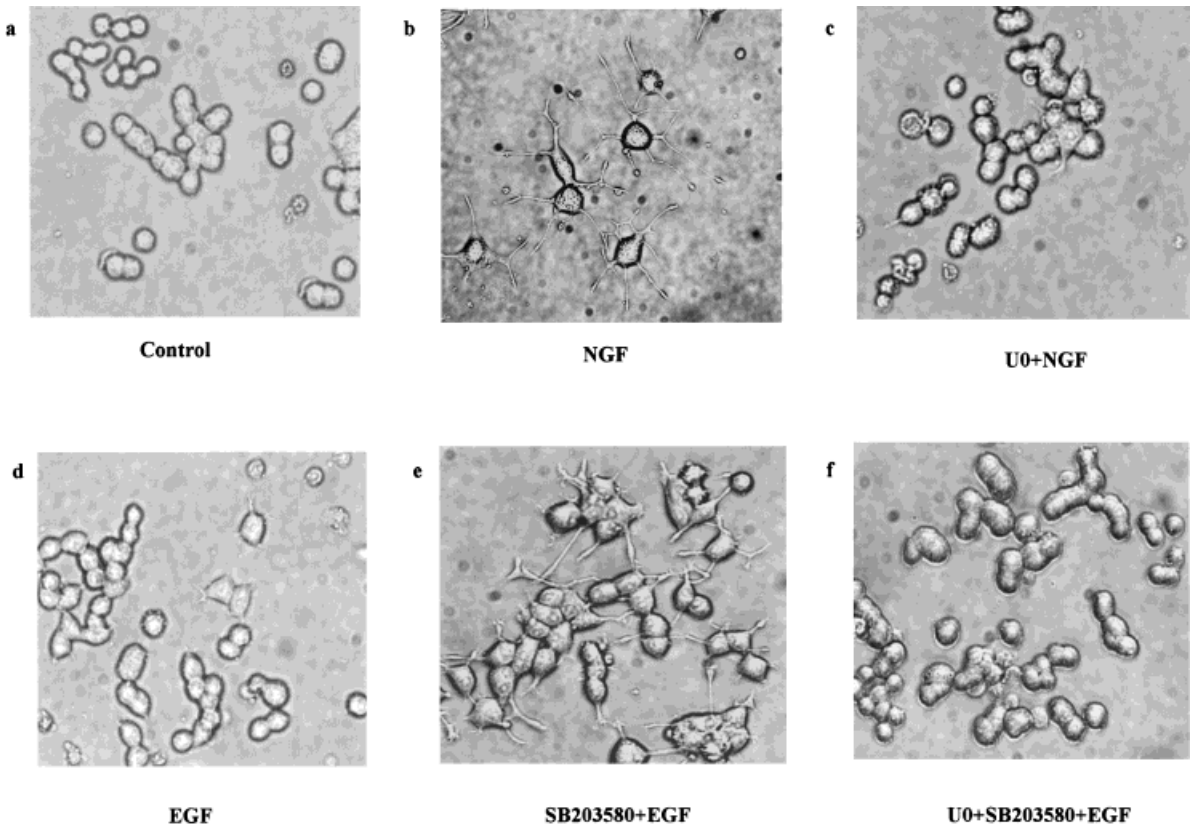

Fig. 6. The effects of U0126 on PC12 cell neurite outgrowth induced by NGF and EGF plus SB203580. The morphological neurite outgrowth from PC12 cells under different treatments are compared: PC12 cells in normal growth medium **(a)**; NGF (100 ng/ml) **(b)**; U0126 (20M) + NGF (100 ng/ml) **(c)**; EGF (200 ng/ml) **(d)**; SB203580 (10 M) + EGF (200 ng/ml) **(e)**; and U0126 (20 M) + SB203580 (10 M) + EGF (200 ng/ml) **(f)**. U0126 and SB203580 were added 30 min before NGF or EGF. The phase-contrast photographs were taken 3 days after treatments.

differences between different laboratories at this moment. It is possible that the PC12 sublines used by different laboratories differs in their responses to SB203580, particularly in regard to ERK activation. In support of this, we observed that both U01216 and SB203580 caused significant apoptosis after 2 days in a low mitogen medium (1% horse serum). The survival of PC12 cells in the presence of SB203580 was much improved in 10% FBS medium (data not shown). The sensitivity of cells to inhibition of the p38 pathway became even more evident when SB203580 was added to the dominant negative p38-expressed PC12 cells (over 80% of the cells would die within 24 h in presence of 10% FBS, data not shown). p38 activity may be required for the survival of newly generated post-mitotic neurons as reported [Mao et al., 1999]. In contrast, cell death was not reported in the studies by Morooka and Nishida [1998] and Iwasaki et al. [1999] in which low mitogenic medium plus SB203580 was used, suggesting different properties in the cells. Although we did not observe significantly direct interplay between p38 and ERK pathways in PC12 cells, the cumulative effect of different MAP kinase activities may be crucial in determining cell differentiation or proliferation.

Threshold Measurement of ERK Activity as a Differentiation Signal in PC12 Cells

Previously, several experimental results have suggested that the sustained activation of ERK by NGF stimulation, as distinct from the transient activation by EGF, may underlie the different abilities of the two factors to induce PC12 cell differentiation [Traverse et al., 1992; Papavassiliou et al., 1995]. Based on these results, a threshold model, which measures both intensity and duration of ERK activity to determine differentiation, has been proposed [Hill and Treisman, 1995]. Other experiments have provided evidence to support this model.

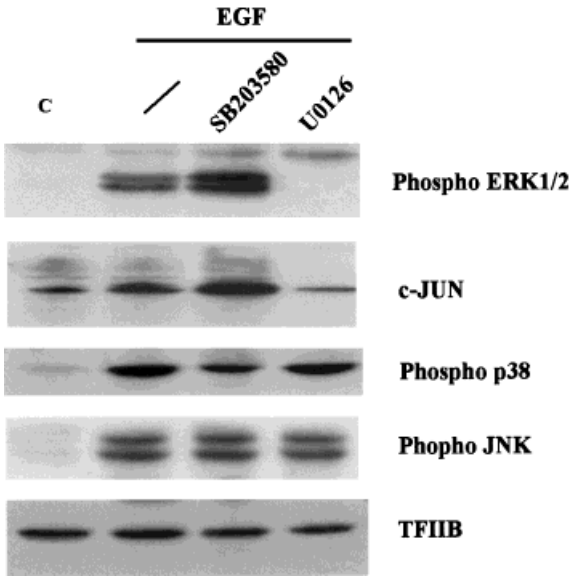

Fig. 7. Comparison of the effects of SB203580 and U0126 on EGF-stimulated activation of ERK1/2, p38 and JNK1/2, and expression of c-Jun in PC12 cells. All but the control PC12 cells were stimulated by EGF for 30 min, whereas indicated the two inhibitors were added 30 min before EGF treatment. The cell lysates were separated on SDS–PAGE and subjected to Western analysis by using different anti-phospho-MAP kinase antibodies or anti-c-Jun antibody.

For example, EGF has been found to trigger differentiation in cells that overexpress EGF receptor, where ERK1/2 activity was enhanced and prolonged [Traverse et al., 1994]; and transgenic expression of PDGF (platelet-derived growth factor) receptor, which was not normally expressed in PC12 cells, conferred PDGF-dependent neuronal differentiation. This may result from the activated tyrosine kinase of PDGF receptor causing sustained ERK1/2 activation [Lai et al., 1990]. Our data support the notion that strength and duration of ERK activation determines PC12 cell differentiation.

Leppä et al. [Minden et al., 1994] have used transfection and microinjection of exogenous MEK1 and c-Jun expression constructs into PC12 cells to show that c-Jun is a downstream substrate of the ERK pathway in PC12 cells. In this system, overexpression of c-Jun caused differentiation without requiring any upstream activators. This work further dissected terminal components in the differentiation signal pathway. c-Jun has long been characterized as a critical transcription factor involved in multiple cellular regulatory processes. Regulation of c-Jun expression has been observed at different levels including transcription [Batistatou et al., 1992], mRNA stability [Wu et al., 1989], protein phosphorylation or dephosphorylation, and ubiquitin-mediated degradation [Baichwal and Tjian, 1990; Minden et al., 1994; Musti et al., 1997]. The elaborate and complex molecular mechanisms regulating c-Jun in response to various extracellular stimuli reflect the importance of this signal in controlling subsequent downstream events. Our experiments demonstrate that the intrinsically distinctive profile of duration of c-Jun expression by NGF or EGF correlates very well with to their abilities to induce PC12 cell differentiation. The level and duration of ERK and p38 activation correlate well with c-Jun induction. Although inhibition of p38 and enhancement of ERK activation by SB203580 should have opposite effect on c-Jun induction [Han et al., 1997; Leppa et al., 1998], the overall effect of this drug on c-Jun expression is of enhancement (Fig. 5B). Therefore, our result is consistent with the proposal that level of c-Jun induction determines cell differentiation.

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