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Dual control of neurite outgrowth by STAT3 and MAP kinase in PC12 cells stimulated with interleukin-6

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IL-6 induces differentiation of PC12 cells pretreated with nerve growth factor (NGF). We explored the signals required for neurite outgrowth of PC12 cells by using a series of mutants of a chimeric receptor consisting of the extracellular domain of the granulocyte-colony stimulating factor (G-CSF) receptor and the cytoplasmic domain of gp130, a signal-transducing subunit of the IL-6 receptor. The mutants incapable of activating the MAP kinase cascade failed to induce neurite outgrowth. Consistently, a MEK inhibitor, PD98059, inhibited neurite outgrowth, showing that activation of the MAP kinase cascade is essential for the differentiation of PC12 cells. In contrast, a mutation that abolished the ability to activate STAT3 did not inhibit, but rather stimulated neurite outgrowth. This mutant did not require NGF pretreatment for neurite outgrowth. Dominant-negative STAT3s mimicked NGF pretreatment, and NGF suppressed the IL-6-induced activation of STAT3, supporting the idea that STAT3 might regulate the differentiation of PC12 cells negatively. These results suggest that neurite outgrowth of PC12 cells is regulated by the balance of MAP kinase and STAT3 signal transduction pathways, and that STAT3 activity can be regulated negatively by NGF.

Keywords: differentiation/interleukin-6/NGF/PC12 cells/STAT3

Introduction

PC12 cells have been used as a model system for the differentiation of neuronal cells. After stimulation with nerve growth factor (NGF), they stop growing, form processes, and exhibit other markers characteristics of neurons such as electrical excitability after appropriate stimuli and formation of synaptic-like vesicles (Greene and Tischler, 1976). It is well known that the ras-MAP kinase, phospholipase C (PLC) and phosphatidylinositol(PI) 3-kinase cascades are activated after NGF treatment (Kim *et al.*, 1991). For complete differentiation of PC12 cells, the presence of NGF throughout the process is required, suggesting that the cells receive the signal from NGF continuously. We have hypothesized that the differentiation procedure may be separated into an early step and the following step, and we screened for the factor capable of inducing neurite outgrowth in PC12 cells only if the cells were pretreated with NGF. We purified one factor and identified it as interleukin-6 (IL-6) (Ihara *et al.*, 1996), a multifunctional cytokine acting in the immune system, haematopoiesis and in inflammation, as well as in neuronal cells (Hirano, 1992; Patterson and Nawa, 1993). IL-6 has been reported to induce neurite outgrowth in PC12 cells (Satoh *et al.*, 1988). However, this effect is limited to certain lines of PC12 cells; most PC12 cell lines do not differentiate after IL-6 stimulation (Ihara *et al.*, 1996; Wu and Bradshaw, 1996a). Using PC12 cells that already extended neurites upon NGF treatment, Wu and Bradshaw (1996a) have shown that IL-6 can induce regeneration of neurites after their removal by shaking the cells. Therefore, it appears that IL-6 can reorganize the materials for neurite formation after cells were completely differentiated. We found that IL-6 can induce differentiation of PC12 cells if they were pretreated with NGF for 1 hour (Ihara *et al.*, 1996). In our system, treatment with NGF is extremely brief compared with the regeneration system, which requires incubation for several days with NGF. The NGF treatment in our system does not allow the cells to undergo most of the process of differentiation, including expression of genes required for the formation of the neurites. We define this NGF-pretreated status as 'initiation'. The IL-6 receptor consists of a ligand recognition subunit, α chain, and a signal-transducing subunit, gp130 (Taga *et al.*, 1989; Hibi *et al.*, 1990). Upon binding of IL-6 to the α chain, the latter associates with gp130, and signals are generated through the cytoplasmic domain of gp130 (Hibi *et al.*, 1990). Jak tyrosine kinases which constitutively associate with the membrane-proximal region of gp130 are activated, resulting in phosphorylation of tyrosine residues located in the cytoplasmic region of gp130 (Lütticken *et al.*, 1994). These phosphotyrosines recruit at least two SH2-containing signalling molecules, SHP-2 (a tyrosine phosphatase also referred as PTP-1, SHPTP-2, PTP2C or Syp) and STAT3 (a signal transducer and activator of transcription) (Sadowski *et al.*, 1993; Stahl *et al.*, 1995; Fukada *et al.*, 1996). Gp130 has six tyrosine residues, five of which in the carboxy-terminus were phosphorylated after ligand stimulation. The second tyrosine from the membrane is responsible for activation of the MAP kinase cascade through SHP-2, and any one of four tyrosines (from the third to the sixth) containing the motif YXXQ is available for STAT3 activation (Bennett *et al.*, 1994; Stahl *et al.*, 1995; Fukada *et al.*, 1996; Yamanaka *et al.*, 1996). Gp130 is a common subunit among the receptors for the IL-6-related cytokine subfamily that includes leukaemia inhibitory factor (LIF) and ciliary neurotrophic factor (CNTF) (Hirano *et al.*, 1994). Both CNTF and LIF promote the transition from noradrenergic to cholinergic function in cultured sympathetic neurons, and

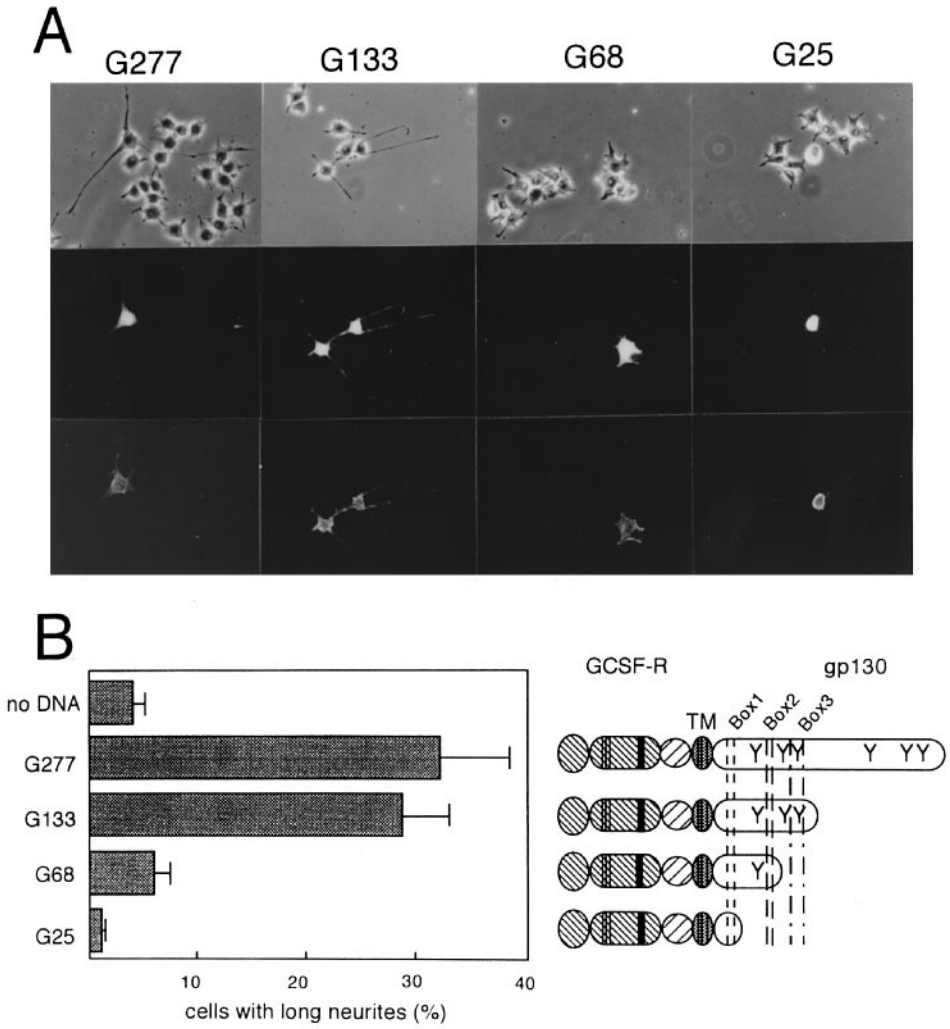

Fig. 1. Induction of neurite outgrowth by G-CSF stimulation in PC12 cells microinjected with G-CSFR–gp130 chimeric receptors. PC12 cells were microinjected with expression vectors for wild-type (G277) or mutant-type G-CSFR-gp130 chimeric receptors (100 ng/ml). After 24 h, cells were initiated with NGF (20 ng/ml) for 2 h and then stimulated with G-CSF (50 ng/ml). After 24 h, the cells were fixed and stained with antibody against the extracellular domain of G-CSF receptor. (**A**) Cells were observed under the microscope with phase contrast (upper panels), by fluorescence of rhodamine–dextran co-microinjected with the expression vectors (middle panels), or by fluorescence of FITC for detection of the chimeric receptors expressed on the cell surface (lower panels). (**B**) Left: cells with long neurites (twice cell length) were counted and normalized to the microinjected (rhodamine-positive) cell numbers. Error bars show standard deviation for three experiments. Right: structures of chimeric receptors are indicated.

they affect the survival and differentiation of motor and sensory neurons (Sendtner *et al.*, 1990; Murphy *et al.*, 1991; Patterson and Nawa, 1993). These results suggest that signal transduction through gp130 may play an important role in neuronal cells in some cellular responses. However, the role of each signal generated through the gp130-containing receptors has not been examined. In this paper, we explored the signals involved in differentiation of PC12 cells by IL-6 stimulation to understand the role of signals mediated via gp130.

Results

Induction of neurite outgrowth through gp130 in PC12 cells

To determine the roles of the signals generated by IL-6 receptor in the differentiation of PC12 cells, we used the G-CSFR–gp130 chimeric receptor (Fukada *et al.*, 1996). This receptor consists of an extracellular domain derived from G-CSFR and a transmembrane domain, as well as an intracellular domain from gp130.

We first tested the effect of G-CSF on PC12 cells initiated with NGF. The cells did not exhibit any morphologic change, suggesting that PC12 cells do not respond to G-CSF (data not shown). The cells were microinjected with the expression vector for the G-CSFR–gp130 chimeric receptor. They did not differentiate when treated with G-CSF alone; however, stimulation with G-CSF after pretreatment with NGF for 2 h resulted in extension of neurites that were indistinguishable from those induced by IL-6 stimulation (Figure 1A). This was consistent with the result that PC12 cells were differentiated by sequential treatment with NGF and IL-6, and suggested that the G-CSFR–gp130 chimeric receptor induces the same cell responses as does the IL-6 receptor. We therefore tested a series of deletion mutants to determine which region of gp130 was required for cell differentiation. Cells were microinjected with the expression vectors for the wild or

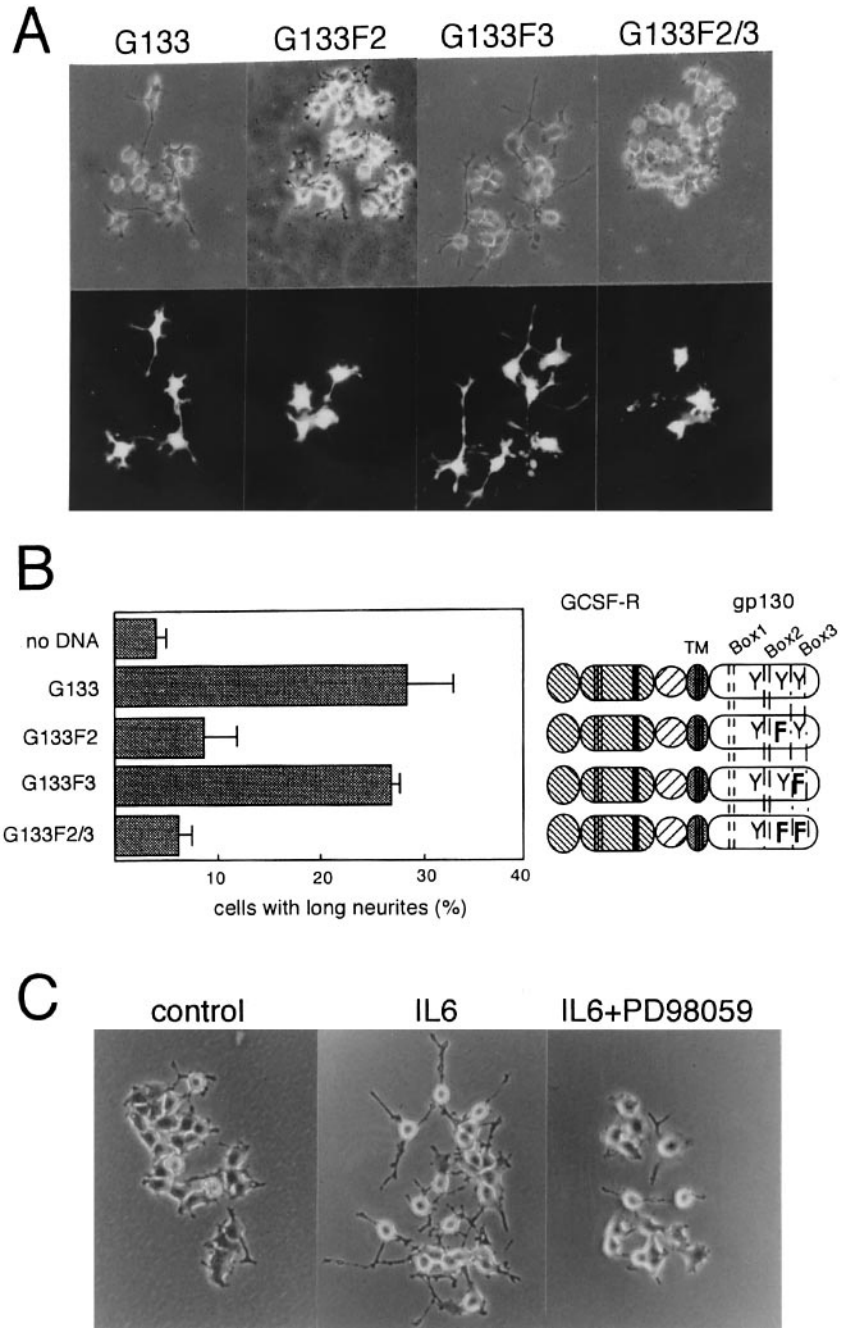

Fig. 2. Activation of MAP kinase cascade is required for neurite outgrowth by IL-6. (**A**) PC12 cells were microinjected with expression vectors for G133 or its derivatives (100 ng/ml). After 24 h, cells were initiated with NGF (20 ng/ml) for 2 h and then stimulated with G-CSF (50 ng/ml). Cells were observed under microscope with phase contrast (upper panel) or by fluorescence of rhodamine–dextran co-microinjected with the expression vectors (lower panels). (**B**) Left: cells with long neurites (twice cell length) were counted and normalized to the microinjected (rhodamine-positive) cell numbers. Error bars show standard deviation for three experiments. Right: structures of the chimeric receptors are shown. (**C**) PC12 cells were initiated with NGF (20 ng/ml) for 2 h in serum-free medium. After removal of the medium, cells were stimulated with serum-free medium containing IL-6 (20 ng/ml) (indicated by IL6 in the figure), or IL-6 (20 ng/ml) with the MEK inhibitor PD98059 (20 μM) (IL6+PD98059). No IL-6 was given to control cells. Cells were observed after incubation for 24 h.

mutant receptors, and the effect of G-CSF after pretreatment with NGF was monitored. As shown in Figure 1, expression of all of the deletion mutants was confirmed by immunofluorescence with anti-G-CSFR antibody. Gp130 still exhibited the activity that induced neurite outgrowth after truncation to amino acid 133 (G133); however, truncation to amino acid 68 (G68) abolished the activity, although cells survived for a few days in the presence of G-CSF activity in serum-free medium (data not shown). Cells expressing a mutant bearing a larger truncation to the 25th amino acid did not respond to G-CSF at all (data not shown). These results suggest that the region between the 26th and 68th amino acids has some role in cell survival, and that the region between the 69th and 133rd amino acids is important for neurite outgrowth of PC12 cells.

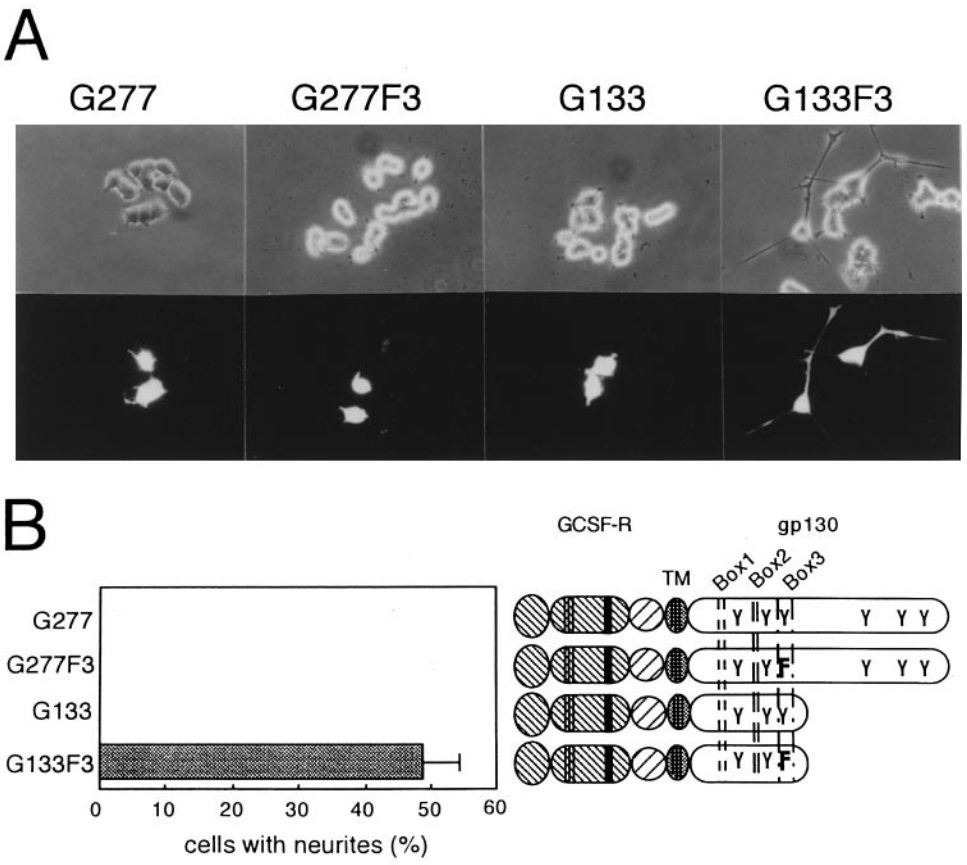

Fig. 3. NGF initiation is not required for neurite outgrowth in PC12 cells microinjected with G133F3. PC12 cells were microinjected with expression vector for G277, G133, or their derivatives (100 ng/ml). After 24 h, cells were stimulated with G-CSF (50 ng/ml) and incubated for another 24 h. (**A**) Cells were observed under the microscope with phase contrast (upper panels) or by fluorescence of rhodamine–dextran co-microinjected with the expression vectors (lower panels). (**B**) Left: cells with neurites longer than the cell length were counted and normalized to the microinjected (rhodamine-positive) cell numbers. None of the cells injected with G277, G277F3 or G133 bore neurites. Error bars show standard deviation for three experiments. Right: structures of the chimeric receptors are indicated.

Activation of the MAP kinase cascade, but not STAT3, is required for neurite outgrowth of PC12 cells initiated with NGF

To determine which signal is important for the neurite outgrowth of PC12 cells, we used the derivatives of a mutant G133, which was the smallest fragment of the G-CSFR–gp130 chimeric receptor capable of inducing neurite outgrowth of PC12 cells. One of the derivatives, G133F2, with a point mutation of the second tyrosine to phenylalanine and lacking the ability to activate the MAP kinase cascade (Fukada *et al.*, 1996), failed to induce neurite outgrowth (Figure 2A and B). Consistent with this result, PD98059, an inhibitor for MAP kinase kinase (Pang *et al.*, 1995), also inhibited neurite outgrowth (Figure 2C). These results suggest that activation of the MAP kinase cascade is required for neurite outgrowth. In contrast, G133F3, which was incapable of activating STAT3, exhibited rather stronger activity in inducing neurite outgrowth than did G133 (Figure 2A). G133F2/3, lacking both of the phosphorylation sites for activation of MAP kinase and STAT3, did not induce any cellular response (Figure 2A and B). These results showed that the activation of MAP kinase, but not of STAT3, was essential for neurite outgrowth of NGF-initiated PC12 cells through gp130.

NGF initiation is not necessary for gp130-mediated neurite outgrowth if STAT3 activation is suppressed

G133F3 appeared to be more effective than G133 or G277 in inducing neurite outgrowth of PC12 cells. Although the total cell number bearing neurites was not changed (Figure 2B), the length of the neurites in G133F3-injected cells was slightly longer than that in G133-injected cells (Figure 2A). It was possible that some negative signal could be induced through the third tyrosine, which might be suppressed by NGF initiation. We tested whether NGF initiation was still required for neurite outgrowth in G133F3-microinjected cells. As shown in Figure 3, G133F3 did not require NGF initiation. However, G277F3, which contains the same mutation at the third tyrosine residue as does G133F3, required initiation. Because other tyrosine residues located in the carboxy-terminus of gp130 containing the YXXQ motif could be a docking site of STAT3 and are sufficient to activate STAT3, this result suggests that STAT3 negatively regulates neurite outgrowth in PC12 cells, and that NGF initiation may suppress STAT3 activation.

Inactivation of STAT3 mimics the effect of NGF

To confirm this idea, we used the dominant-negative mutants of STAT3, STAT3D and STAT3F (Nakajima *et al.*,

1996). These mutant STAT3s were co-expressed with G277, which required NGF initiation. As shown in Figure 4A and B, PC12 cells differentiated without NGF initiation after expression of the dominant-negative STAT3s. For confirmation that these dominant-negative STAT3s suppressed STAT3 activity in this system, a reporter plasmid carrying *lacZ* genes driven by the acute-phase response element (APRE), a response element for STAT3 derived from the rat α2-macroglobulin promoter (Wegenka *et al.*, 1993), was microinjected together with G277 and the dominant-negative STAT3 mutants. In this system, induction of the *lacZ* gene by STAT3 was monitored by cleavage of X-gal by β-galactosidase. As shown in Figure 4C, the cells extending neurites by the expression of STAT3F or STAT3D exhibited little or faint blue colour, whereas those without expression of the dominant-negative STAT3s were stained intensely. This result suggests that STAT3 activity was lower in the cells extending neurites. Taken together, these findings suggest that suppression of STAT3 enables IL-6 to induce neurite outgrowth of PC12 cells, and that NGF initiation might suppress STAT3 activation. This possibility was tested. After microinjection of the reporter plasmid and G277, cells were stimulated with G-CSF. In the absence of NGF, 75% (49 of 65) of the microinjected cells showed a blue colour, indicating that STAT3 was activated in these cells. In contrast, stimulation with G-CSF in the presence of NGF induced STAT3 activation in only 25% (16 of 65) of the cells. NGF did not cause any activation of STAT3 (data not shown).

We further examined whether NGF initiation can inhibit IL-6-induced tyrosine phosphorylation of STAT3 in PC12 cells. As shown in Figure 5, STAT3 was clearly tyrosine-phosphorylated after IL-6 stimulation. Treatment with NGF for more than 1 h resulted in less phosphorylation. Treatment for 30 min showed no effect. These results suggest that NGF treatment for more than 1 h suppressed the IL-6-induced activation of STAT3, although the expression level of STAT3 was not changed. This result was consistent with the fact that the establishment of initiation by NGF requires at least 1 h, supporting the notion that inactivation of STAT3 can be the major effect of NGF initiation (Figure 5B). Pretreatment of the cells with EGF did not inhibit the activation of STAT3 by IL-6 stimulation (Figure 5A, lanes 8 and 9).

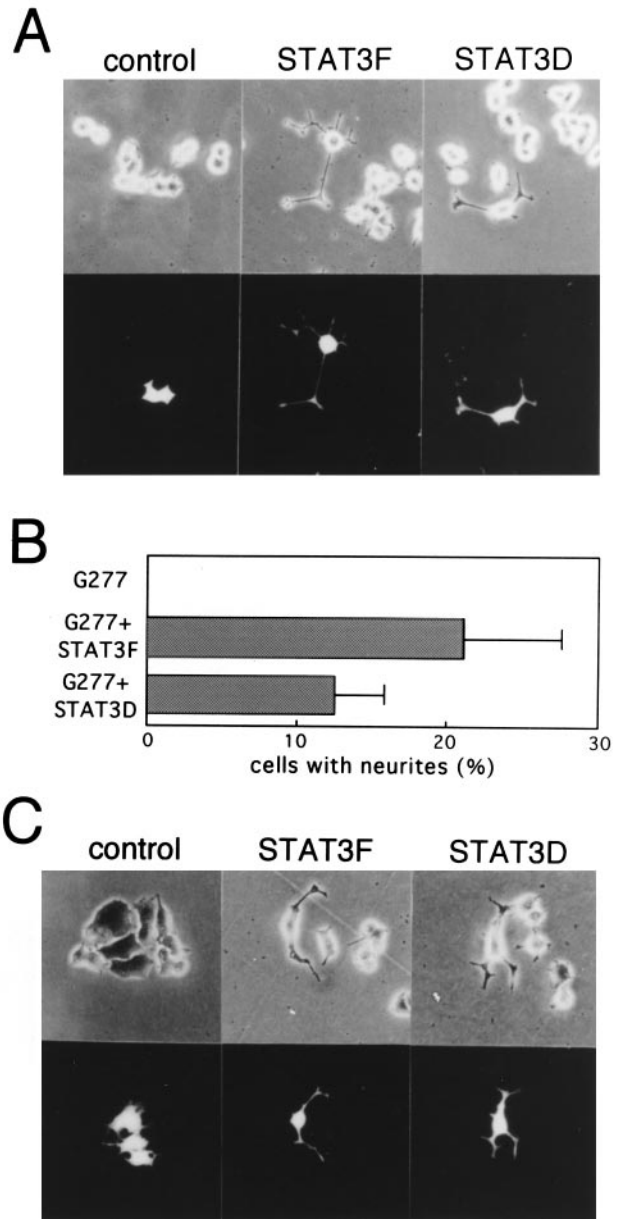

Fig. 4. The effect of dominant-negative STAT3s on neurite outgrowth of PC12 cells microinjected with wild-type G277. (**A**) PC12 cells were co-injected with expression vectors for G277 (50 ng/ml) together with dominant-negative STAT3s, STAT3F and STAT3D, or with the control vector (200 ng/ml). After 24 h, cells were stimulated with G-CSF (50 ng/ml) and incubated for 24 h. Cells were observed under the microscope with phase contrast (upper panels) or by fluorescence of rhodamine–dextran co-microinjected with the expression vectors (lower panels). (**B**) Cells with neurites (longer than their cell length) in (A) were counted and normalized to the microinjected (rhodamine-positive) cell numbers. None of the cells without expression of dominant-negative STAT3 bore neurites. The efficiency of the neurite outgrowth in the cells injected with the combination of dominant-negative STAT3 and G277 was less than the efficiency for those injected with G133F3: the number of cells with neurites was smaller, and neurites were shorter. Because suppression of STAT3 activity by the dominant-negative STAT3s was not complete, exhibiting low activity of β-galactosidase with a longer incubation time, it is conceivable that the limited STAT3 activity remaining in the cells may partially inhibit neurite outgrowth. Error bars show standard deviation for three experiments. (**C**) PC12 cells were co-microinjected with a mixture of G277 (40 ng/ ml), dominant-negative STAT3s (160 ng/ml), and 4×APRELacZ (50 ng/ ml). The empty vector (160 ng/ml) was used as a control for dominant-negative STAT3s. After 24 h, cells were fixed and assayed for expression of the *lacZ* gene by X-gal. Cells were observed under the microscope with phase contrast (upper panels) or by fluorescence of rhodamine–dextran co-microinjected with the expression vectors (lower panels).

Discussion

It has been suggested that IL-6 regulates cell growth and differentiation not only in lymphocytes, but also in neuronal cells (Hirano, 1992; Patterson and Nawa, 1993; Hirano *et al.*, 1994). For example, production of IL-6 by glioblastoma cells or astrocytoma cells stimulated by IL-1 has been reported (Yasukawa *et al.*, 1987), suggesting that IL-6 may be present in the environment of neuronal cells. It has also been demonstrated that IL-6 can support the survival of cultured cholinergic neurons (Hama *et al.*, 1989). These findings strongly suggest that IL-6 has some

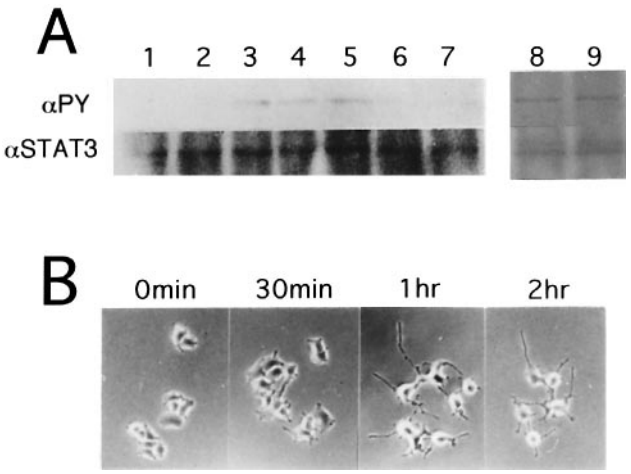

Fig. 5. Effects of the period of NGF initiation on the suppression of STAT3 activation by IL-6 stimulation. (**A**) After preincubation with NGF for 0 min (lane 4), 30 min (lane 5), 1 h (lane 6) and 2 h (lane 7), or with EGF for 0 min (lane 8) and 2 h (lane 9); IL-6 was added and incubated for 15 min. Cells were lysed and immunoprecipitated with anti-STAT3 antibody. The precipitates were immunoblotted with anti-phosphotyrosine antibody (4G10), and detected by the ECL method. Lanes 1–3 are controls. Lane 1, no stimulation; lane 2, stimulated with NGF for 15 min; lane 3, IL-6 for 15 min. (**B**) PC12 cells were preincubated with NGF for the indicated periods. The medium was then replaced with serum-free medium containing IL-6 (20 ng/ml). At 24 h after the IL-6 stimulation, cells were observed under the microscope.

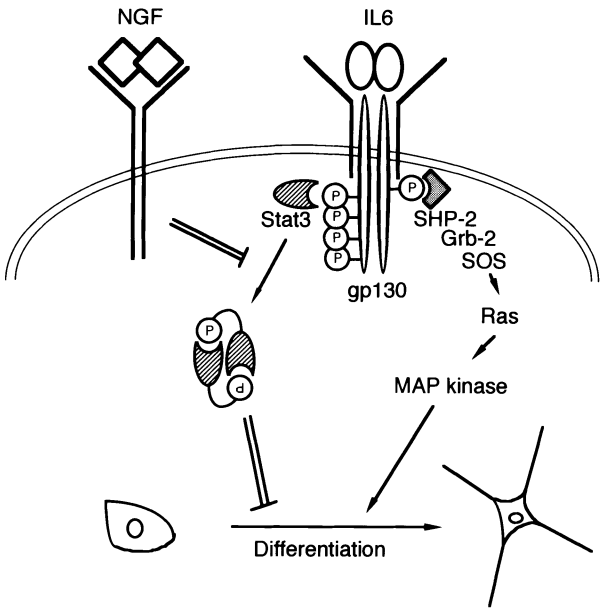

Fig. 6. Schematic model of signalling through gp130, required for neurite outgrowth by IL-6. Two signalling pathways, MAP kinase and STAT3, are involved in the differentiation of PC12 cells induced by IL-6. Activation of MAP kinase is required for the cellular response. In contrast, the STAT3 cascade negatively regulates differentiation of PC12 cells. NGF can work as a suppressor of STAT3, which enables the NGF-initiated cells to differentiate by stimulation with IL-6.

role in neuronal development. IL-6 was shown to induce differentiation of PC12 cells (Satoh *et al.*, 1988), though such effect was limited to some PC12 cell sublines. Bradshaw and colleagues recently showed that most of the PC12 cell lines do not differentiate after IL-6 stimulation (Wu and Bradshaw, 1996a). We found that IL-6 could induce differentiation of PC12 cells, provided that the cells were pretreated with NGF (Ihara *et al.*, 1996). Using this system, we explored the roles of signals generated by IL-6.

We found that blockade of the MAP kinase cascade inhibited the differentiation of PC12 cells. Loss of a signalling site required for activation of the MAP kinase cascade on gp130, or inhibition of MAP kinase kinase by an inhibitor gave virtually the same results, indicating that activation of the MAP kinase pathway is required for neurite outgrowth. It is well known that constitutive activation of the MAP kinase cascade by activated Ras or MAP kinase kinase induces neurite outgrowth of the cells (Noda *et al.*, 1985; Cowley *et al.*, 1994). Our finding is consistent with the previous results that NGF uses the MAP kinase cascade to induce neurite outgrowth. In contrast, however, our results suggested that STAT3 can be a negative regulator of neurite outgrowth. Suppression of STAT3 activation by a point mutation in truncated gp130 or by the expression of dominant-negative STAT3 mutants allowed IL-6 to induce differentiation of PC12 cells without NGF initiation. Finally, we found that activation of STAT3 was suppressed by NGF. Stimulation with IL-6 alone did not induce differentiation of PC12 cells, probably because of strong activation of STAT3. NGF may release this suppression of differentiation by inactivating STAT3. Possibly, the STAT3 signalling pathway may be blocked in some way in the PC12 cell lines which differentiate by stimulation with IL-6 in the absence of NGF. Our preliminary results suggest that treatment with vanadate inhibits suppression of STAT3 activation by NGF. It has been reported that SHPTP-1 is activated in PC12 cells stimulated by NGF (Vambutas *et al.*, 1995). Another report suggests that a protein tyrosine phosphatase, PTP20, can be a positive regulator of differentiation in PC12 cells (Aoki *et al.*, 1996). It may be interesting to speculate that these phosphatases might dephosphorylate STAT3 in order to inactivate it. NGF could also have effects other than suppression of STAT3. However, our findings suggest that the neurite outgrowth of PC12 cells may be regulated by the balance of two signalling pathways, the MAP kinase and STAT3 pathways (Figure 6). Wu *et al.* have suggested that, rather than the MAP kinase cascade, it is STAT3 that might be important for regeneration of neurites by IL-6 (Wu and Bradshaw, 1996a,b). It is possible that the signals required for regeneration and *de novo* formation of neurites are different.

It should be noted that EGF, which induces the growth of PC12 cells, did not suppress the activation of STAT3 (Figure 5). Most of the second messengers generated by EGF and NGF stimulation are similar, although activation periods of the MAP kinase cascade and PI3 kinase are of longer duration in NGF stimulation than in EGF stimulation. Among these second messengers, STAT3 is the one of the molecules whose behaviour contrasts between NGF and EGF stimulation. In a mouse myeloid leukaemic cell line, M1, STAT3 activation is essential for IL-6-induced macrophage differentiation and growth arrest (Nakajima *et al.*, 1996; Yamanaka *et al.*, 1996) whereas in a mouse pro-B-cell line, BAF-B03, activation of STAT3 is required for cell survival (Fukada *et al.*, 1996). These facts suggest

that STAT3 plays a critical role in determination of the cell fate.

The question is then, how can IL-6 work in neuronal system *in vivo*? Many neuronal cells may be stimulated with some neurotrophins at some stage. Thus, primary neuronal cell cultures may have been exposed to these factors before explantation, which might mean that STAT3 is already suppressed. In fact, IL-6 can induce neurite outgrowth of neuronal cells cultured from rat or chicken embryos without initiation with NGF (Hama *et al.*, 1989). These findings indicate that IL-6 may stimulate neuronal cells if they are initiated with some neurotrophins *in vivo*, though further study may be required to substantiate this idea.

Materials and methods

Plasmids

The plasmids bearing the genes that encode chimeric proteins of the extracellular domain of G-CSFR and the transmembrane and cytoplasmic domains of gp130 were as described before (Fukada *et al.*, 1996). A reporter plasmid for detection of the activity of STAT3, 4×APRELacZ, was constructed by joining of the β-galactosidase gene with four repeats of APRE and junB minimal promoter derived from a plasmid, 4×APRELuc (Nakajima *et al.*, 1995). The expression vectors for dominant-negative STAT3 were described previously (Nakajima *et al.*, 1996).

Cell culture and microinjection

Rat phaeochromocytoma PC12 cells were cultured in Dulbecco's modified minimal essential medium (DMEM) supplemented with 10% calf serum and 5% horse serum on coverslips coated with poly-L-lysine. These cells were microinjected with the plasmids dissolved in phosphate-buffered saline (PBS) containing rhodamine–dextran (2.5 mg/ml). The cells were treated with NGF (20 ng/ml) for 2 h. Cells were washed twice with serum-free DMEM and then stimulated with G-CSF (50 ng/ml) and incubated for 24 h at 37°C. The injected cells were observed under the microscope. In each experiment, ~100 rhodamine-positive cells were monitored for morphological changes. The experiment was done three times, and the average determined.

Detection of STAT3 activity by the reporter plasmid containing the lacZ gene

After fixation with 2% formalin and 0.2% glutaraldehyde for 5 min at 4°C, the cells were incubated with a buffer containing 1 mg/ml X-gal (5-bromo-4-chloro-3-indolyl-D-galactoside), 5 mM potassium ferricyanide, 5 mM potassium ferrocyanide and 2 mM $MgCl_2$ for 1 h at 37°C.

Staining of G-CSFR–gp130 chimeric receptors expressed on the cell surface

The cells were fixed for 20 min at room temperature by addition of formalin to the medium (final concentration 10%). After blocking with DMEM containing 10% calf serum, the cells were incubated with an antibody against the extracellular domain of G-CSFR (Fukada *et al.*, 1996) at room temperature for 60 min in a blocking buffer, and then with FITC-conjugated anti-rabbit antibody (goat) as a second antibody for 60 min.

Immunoprecipitation and Western blotting

Aliquots of 1×10^7 cells stimulated with the growth factors were lysed in NP40 buffer (50 mM Tris–HCl, pH 7.5, 150 mM NaCl, 1 mM EDTA, 0.5% NP40, 1 mM sodium orthovanadate, 1 mM phenylmethylsulfonylfluoride, 15% glycerol). The STAT3 protein was immunoprecipitated with anti-STAT3 antibody (Kojima *et al.*, 1996) bound to protein A–Sepharose (Pharmacia). After washing three times with the NP40 buffer, the phosphorylation of STAT3 on tyrosine was detected by Western blotting with anti-phosphotyrosine antibody, 4G10 (UBI). The blocking was done in a buffer containing 3% BSA. The bands were detected by the ECL method (Amersham).

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