Activation of the Stat3 Signaling Pathway Is Required for Differentiation by Interleukin-6 in PC12-E2 Cells*

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Cytokines play an essential role in the regulation of multiple cellular functions including cell proliferation, differentiation, and survival. Most cytokines activate Jak/signal transducer and activator of transcription (STAT) signaling pathways, which are indispensable in the regulation of various cellular programs (1–4). Jak/s are cytoplasmic tyrosine kinase effectors that are activated by ligand-receptor interactions, leading to the tyrosine phosphorylation and activation of the cytoplasmic transcription factors, STATs, which then translocate to the nucleus and act on target gene transcription. To date, genes for four kinases of the Jak family and seven mammalian STATs have been identified, which may be activated individually or in combination (5). However, STAT pathways are not found exclusively in cytokine signal transduction and thus are also important for several growth factor-activated tyrosine kinase receptor-mediated mechanisms.

Recent studies using Jak- or STAT-deficient mice have assigned specific cellular functions to most of these signaling components (6). For example, Jak1 has been shown to be essential for signaling by gp130-containing cytokine receptors (7), both Jak1 and Jak3 are essential in lymphocyte development, and Jak2 deficiency is lethal due to defective erythropoiesis (7–10). Similarly, the STATs also have specific roles in cell regulation. Stat1 is essential for interferon signaling and innate response to viral and bacterial infection (11), while Stat5a is associated with the mammary gland development (6). In contrast, Stat3 is critical for embryonic development; Stat3-deficient mice die before gastrulation (12).

Interleukin-6 (IL-6) belongs to a cytokine subfamily, whose members include ciliary neurotrophic factor, leukemia inhibitory factor, interleukin-11, oncostatin M, and cardiotrophin-1 and which share a common signal transducing molecule, gp130, in their respective receptor complexes (13). Unlike tyrosine kinase receptors, IL-6 receptor α subunit (IL-6Rα) does not possess intrinsic tyrosine kinase activities. Rather, binding of IL-6 to IL-6Rα triggers the association and dimerization of the signal-transducing component gp130.

As with most cytokines capable of inducing multiple cellular functions, accumulating evidence suggests that this subfamily of cytokines signaling through gp130 is involved in neuronal regulation in addition to its well established functions in hematopoiesis, immune, and inflammatory responses. Ciliary neurotrophic factor and leukemia inhibitory factor are involved in the neuronal differentiation and regeneration as well as cell fate determination (14–17). Although not as well established, IL-6 has also been implicated as a potential regulatory agent in the nervous system (18, 19). Both IL-6 and its ligand binding subunit IL-6Rα are expressed and localized in discrete areas of the central nervous system (20). In addition, they are synthesized and secreted in both central and peripheral nervous tissues in response to inflammatory stimuli or other environmental insults (21–26). Moreover, it has been shown that IL-6 prevents cell death and promotes survival in some cerebral neurons as well as cultured sympathetic neurons (21, 27–31).

IL-6 may also play a potential role in neuronal cell differentiation or regeneration (18, 19, 23, 32). We have reported that it is capable of inducing morphological differentiation in PC12 variant E2 cells or in PC12 cells previously exposed to nerve growth factor (NGF) or basic fibroblast growth factor (bFGF) (primed) but not native PC12 cells. Furthermore, a synergistic
induction of neurite proliferation and neuronal specific genes was observed by treatment of either wild type or variant cells with combinations of subthreshold concentrations of IL-6 and NGF or epidermal growth factor (EGF) (33). Recent reports by others also suggest an involvement of IL-6 in morphological differentiation by different PC12 variants under various treatment conditions (34–38).

In previous studies, the differential response of PC12-E2 or primed PC12 cells to IL-6 was shown to be largely independent of the activation of the RAS/ERK pathway (32), which is required for the same response to NGF and bFGF (39, 40). Instead, it was observed that IL-6 predominantly activates Stat3 and to a lesser extent the Stat1 signaling pathway. IL-6 did induce the tyrosine phosphorylation of ERK, but the activation was found to be transient and very weak compared with that produced by NGF, bFGF, or even EGF. In contrast, Ihara et al. (35) have reported that the Stat3 stimulated by IL-6 in PC12 cells pretreated with NGF (1–2 h) is actually inhibitory of differentiation and that the positive responses observed are via gp130-mediated activation of ERKs.

To resolve these conflicting mechanisms and to further evaluate the contributions of these intracellular signaling pathways to IL-6-induced morphological differentiation of PC12-E2 cells, PC12-E2 cells overexpressing dominant negative variants of Stat1, Stat3, and p21/22 were examined. The results clearly demonstrate that activation of the Stat3, and not the RAS/ERK, pathway is involved in the neurite proliferative response to IL-6. Thus, these signaling pathways act in a substantially similar way and are positive stimulators of PC12 cell differentiation.

EXPERIMENTAL PROCEDURES

Cell Culture—Stable PC12 variant E2 cells characterized by more rapid responses to NGF and bFGF and markedly more robust responses to IL-6 were isolated from the parental PC12 line as described previously (41). Cells were maintained as monolayer cultures in 162-mm2 tissue culture flasks (Costar, Cambridge, MA) in Dulbecco’s modified Eagle’s medium containing 10% heat-inactivated horse serum (Life Technologies, Inc.), 5% fetal calf serum and 1% penicillin-streptomycin. Cells were subcultured once a week by trypsinization and replating in a 1:4 to 1:6 ratio. The medium was changed to low serum medium overnight before stimulation with NGF (100 ng/ml for 5 min or by RNase treatment for 5 min). For the effect of MAPKK inhibitor on protein tyrosine phosphorylation of ERK, PC12-E2 cells were pretreated with PD98059 (a gift from Parke-Davis, Ann Arbor, MI) for 1 h before stimulation with NGF or IL-6 as described above. The following antibodies were used in this study: mouse monoclonal anti-FLAG antibody (M5; Eastman Kodak Co.); mouse monoclonal anti-phosphotyrosine (4G10; Upstate Biotechnology, Inc., Lake Placid, NY); anti-pan-ERK monoclonal antibody (Transduction Laboratories, Lexington, KY); rabbit polyclonal anti-GST antibody and anti-Stat3 polyclonal antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA); Anti-active MAPKT polyconal antibody (Promega, Madison, WI); rabbit polyclonal anti-Phospho-Stat3(Ser727) antibody (New England Biolabs, Inc., Beverly, MA); and rat monoclonal anti-p21/22 (259) antibody (Biosource, Camarillo, CA).

Immunocytochemistry—PC12-E2 cells were grown on rat tail collagen-coated glass coverslips in six-well plates and transfected with 1 μg of DNA using Lipofectin reagent (Life Technologies) according to the manufacturer’s instructions. After incubation for 5 h in Opti-MEM (Life Technologies), cells were allowed to recover for 24 h in complete medium and then treated with IL-6 (30 ng/ml) or NGF (100 ng/ml) in Dulbecco’s modified Eagle’s medium containing 1% horse serum (low serum medium) for 40 h.

Immunostaining of PC12-E2 cells following growth factor stimulation was performed essentially as described previously (47). For cells transfected with FLAG-tagged constructs, cells were incubated with anti-FLAG monoclonal antibody (M5) overnight at 4 °C followed by rabbit anti-mouse IgG (Jackson Laboratory, Bar Harbor, ME) for 2 h and fluorescein isothiocyanate-conjugated (FITC) goat anti-rabbit IgG (Molecular Probes, Inc., Eugene, OR) for 2 h at room temperature. Immunostained cells were identified under an epifluorescence-equipped microscope, and responsive cells were scored as cells extending neurites at least two cell bodies in length.

Electrophoretic Mobility Shift Assay (EMSA)—Preparation of crude nuclear extracts and EMSA were performed as described previously (32).

Luciferase Reporter Gene Assays—The Stat1 reporter gene construct, pTAL-LucGAS, was constructed by inserting four copies of an annealed oligonucleotide corresponding to the GAS element from the murine Ly-6E gene (48) into the KpnI and XmnI sites upstream of the thymidine kinase minimal promoter of the luciferase reporter vector pTAL-Luc (Stratagene, CA). The Stat3 reporter gene construct (complete sequence), pLucTRKS3, containing seven copies of the human C-reactive protein gene acute-phase responsive element, was a generous gift from Dr. Jove (H. Lee Moffitt Cancer Center and Research Institute, Tampa, Florida) (49). PC12-E2 cells were grown on rat tail collagen-coated six-well plates and transfected with a mixture of plasmids containing Stat3 or Stat1 reporter constructs (4 μg), pRL-null, a Renilla luciferase expression plasmid (1 μg; Promega), and dominant negative Stat constructs (Stat3 constructs, 4 μg; Stat1 constructs, 2 μg) using Lipofectin reagent (Promega). Luciferase expression was measured 48 h after transfection.

Stable Cell Line Expressing Dominant Negative ras—Dominant negative ras cDNA (v-Ha-ras-Asn17) was subcloned into pCMV-Hygro expression vector (kindly provided by Dr. Eric Stanbridge, University of California, Irvine) and used to transfect PC12-E2 cells using Lipofectin reagent to generate stable cell lines. One day following transfection, cells were seeded onto 10-cm2 flasks (Corning) in Dulbecco’s modified Eagle’s medium (Dulbecco’s modified Eagle’s medium containing 10% fetal bovine serum and 5% horse serum), and selection was carried out for 3 weeks until individual colonies could be selected and screened for p21/22 expression. Viable cell growth was determined by Trypan blue exclusion.
Neurite Outgrowth—The neurite outgrowth assay was performed as described previously (32). For the induction of neurite outgrowth in cells expressing dominant negative RAS proteins, cells were grown in complete medium for 6 h and changed to low serum medium with the addition of IL-6 (30 ng/ml) or NGF (100 ng/ml) for 1–4 days. For the effect of MAPK inhibitor PD98059 on neurite outgrowth, PC12-E2 cells were grown in complete medium for 16 h, changed to low serum medium, and pretreated with PD98059 (0 to 30 μM) for 1 h before the addition of IL-6, NGF, or no factors (control) for 24 h. The percentage of responsive cells was scored.

RESULTS

Construction of Dominant Negative Forms of STAT Mutants—Results from previous studies have suggested that IL-6 stimulates neurite proliferation in PC12-E2 cells by a mechanism that is substantially independent of RAS/ERK and that the response is most likely mediated by activation of Jak/STAT pathways (32). However, Ihara et al. (35) found STAT activation to be inhibitory of PC12 cell differentiation. To better resolve the role of Jak/STAT pathways in IL-6-induced neurite proliferation, various dominant negative mutants of Stat1 and Stat3 proteins were introduced into PC12-E2 cells, and their effects were tested.

The properties and effectiveness of several dominant negative forms of Stat1 and Stat3 have been previously characterized (50–53). In the present study, these inhibitors were prepared by mutating a single tyrosine residue to phenylalanine (YF), a single serine residue to alanine (SA), or a combination of both (DM), using oligonucleotide-directed mutagenesis. The cDNA constructs generated, including wild type rat Stat3 (FS3) and rat Stat1 (FS1) and dominant negative forms (FS3-YF, FS3-SA, FS3-DM, FS1-YF, FS1-SA, and FS1-DM), were tagged at the 5’ end with a FLAG epitope. Expression and integrity of the wild type and mutated STATs were first verified by transiently transfecting each construct into human embryonic kidney 293T cells co-expressing rat IL-6Ra (Fig. 1A). Anti-Stat3 and anti-Stat1 immunoblots of anti-FLAG immunoprecipitates from 293T cell lysates suggest that all constructs were expressed robustly and at a similar level in 293T cells. Anti-phosphotyrosine and anti-phospho-Stat3(Ser727) blots show that stimulation with IL-6 results in tyrosine phosphorylation of FS3 and an increased serine phosphorylation (Fig. 1A). It is also noted that serine phosphorylation of Stat3 proteins results in a reduced mobility of FS3 as compared with FS3-SA shown on the phosphotyrosine blot. When FS3 protein, in which Tyr705 is replaced by phenylalanine (FS3-YF or FS3-DM) was expressed, no tyrosine phosphorylation was detected after IL-6 stimulation. When Ser727 on FS3 protein was replaced by an alanine residue (FS3-SA or FS3-DM), no serine phosphorylation was detected in the presence or absence of IL-6 stimulation. Similarly, FS1 was phosphorylated on tyrosine after IL-6 stimulation (Fig. 1B). However, no tyrosine phosphorylation was detected in FS1 containing a phenylalanine at position 701 (FS1-YF or FS1-DM). Mutation of Ser727 to alanine was confirmed by double-stranded DNA sequencing.

The Effects of Dominant Negative STATs on IL-6-induced Neurite Outgrowth in PC12-E2 Cells—To determine if neurite outgrowth induced by IL-6 is mediated by activation of the Jak/STAT pathway, dominant negative STAT constructs were transiently introduced into PC12-E2 cells. Cells expressing recombinant proteins were identified by immunostaining with anti-FLAG monoclonal antibody, rabbit anti-mouse IgG, and FITC-conjugated goat anti-rabbit antibody and examined under an epifluorescent microscope (Fig. 2). IL-6 and NGF in-
duced 72.2 ± 1.1 and 78.6 ± 2.2% responsive cells in neurite outgrowth assay, respectively, in PC12-E2 cells transfected with the control construct, FPDI (Fig. 3). Overexpressing wild type FS3 does not significantly affect neurite outgrowth induced by either NGF or IL-6. In contrast, neurite outgrowth induced by IL-6 is greatly inhibited in cells overexpressing FS3-YF, but there is no effect on NGF-induced neurite outgrowth. The FS3-SA mutant can be phosphorylated on Tyr705, forming homodimers or dimers with endogenous STATs, and translocated to the nucleus. Although they may bind DNA, the transcriptional efficiency is apparently less than the homodimers of the wild type Stat3. Expression of FS3-SA produces a weak inhibitory effect on IL-6-induced neurite formation (reduced by about 30%), but, as with FS3-YF, NGF-induced neurite outgrowth is not affected. Expression of FS3-DM produces complete inhibition of the IL-6-induced neurite proliferation. Responsive cells were reduced to control level. Again, NGF-induced neurite outgrowth was not affected by the expression of FS3-DM. These results clearly suggest that the activation of Stat3 pathway is essential for IL-6 to induce morphological differentiation of PC12-E2 cells.

Although IL-6 predominantly activates Stat3 in PC12-E2 cells, it transiently activates Stat1 as well (32). To examine if Stat1 plays a role in IL-6-induced neurite outgrowth in these cells, similar experiments using wild type and dominant negative mutants of Stat1 were performed. Interestingly, overexpression of the wild type FS1 protein inhibits IL-6-induced neurite outgrowth by 60% (Figs. 2 and 3). Mutant Stat1 proteins also produce partial inhibition of the IL-6-induced neurite outgrowth. As with wild type and the mutant Stat3 proteins, Stat1 and the dominant negative Stat1 mutants have no effect on NGF-induced neurite outgrowth. It appears that the wild type Stat1 proteins exhibit partial inhibitory effects on IL-6 activated Stat3 signaling. It was previously reported that the major STAT-DNA binding complexes formed in PC12 cells after IL-6 stimulation consist of Stat3 homodimers. Thus, it is likely that overexpressed FS1 proteins alter the pattern of DNA binding complexes in favor of the formation of Stat1 homodimers or Stat1-Stat3 heterodimers.

**The Effect of Dominant Negative STATs on IL-6-induced DNA Binding Activity**—To determine the effect of dominant negative STATs on IL-6-induced *sis*-inducible element (SIE) binding activity, 293T cells were transiently transfected with pCMV-IL-6Ra together with expression vectors for various STAT constructs. EMSAs using 32P-labeled high affinity SIE elements and nuclear extracts from cells treated with IL-6 were performed. Treatment of cells expressing FPDI with IL-6 for 15 min leads to formation of three STAT-DNA complexes, SIFA, -B, and -C (Fig. 4) (54). The slowest migrating complex, SIFA, which contains the heterodimers of Stat1 and Stat3, is less intense. The induction of SIFC, which contains the homodimers of Stat1, is barely detectable. The pattern of DNA binding complexes is altered in cells overexpressing FS1. In these cells, the strongest complex formed after 15 min of IL-6 stimulation is SIFC. The SIFB complex is not as intense as SIFC but is stronger than SIFB observed in cells overexpressing the control, FPDI. The formation of SIFA is inhibited in these cells. The dominant negative mutant of Stat1, FS1-DM, cannot be activated by IL-6 to induce SIE binding activities;
however, the formation of native SIFA and SIFB complexes observed in cells transfected with control construct is modestly reduced (Fig. 4). These results suggest that overexpression of either wild type FS1 or the dominant negative FS1-DM inhibits to some degree IL-6-activated Stat3 DNA binding activities.

In cells overexpressing wild type Stat3, the migration of the SIFA complex containing FS3 homodimers is slightly faster, and the intensity of this complex is modestly increased as compared with native SIFA complexes. The FS3-DM is a potent dominant negative inhibitor for IL-6-activated Stat3 signaling. Treatment of cells expressing FS3-DM with IL-6 does not stimulate any DNA binding activity. These results support the view that Stat3 homodimers are most likely the entities required for the induction of differentiation-related genes by IL-6 and that a Stat3 but not a Stat1 signaling pathway is preferentially utilized by IL-6 to induce neurite proliferation in E2 cells.

**The Effects of Dominant Negative STATs on IL-6-induced Transcriptional Activities**—The luciferase reporter gene assay were performed to further substantiate the transcriptional efficiencies of dominant negative STATs in PC12-E2 cells. IL-6 does not induce luciferase activity in cells transiently transfected with control constructs pLucTK or pTAL-Luc. However, the luciferase activity was increased by 2.8-fold in cells transiently transfected with the Stat3 reporter plasmid pLucTKS3, containing seven copies of Stat3-specific binding sites, from the human C-reactive protein gene acute-phase responsive element (49) (Fig. 5A). Co-expressing wild type FS3 modestly enhanced IL-6-induced transcription. In contrast, luciferase activity induced by IL-6 was reduced to basal level in cells co-expressing FS3-YF or FS3-DM. Expression of FS3-SA also modestly reduced IL-6-induced luciferase activity, which is consistent with its inhibitory effect on IL-6-induced neurite outgrowth. These data confirmed that inhibition of neurite outgrowth by Stat3 mutants indeed results from inhibition of Stat3-dependent transcription.

The results described above suggest that expression of wild type FS1 may inhibit IL-6-activated transcription. However, most STAT proteins have similar DNA binding preferences; therefore, this possibility cannot be effectively tested using the artificial reporter plasmid, pLucTKS3. Since we have reported that IL-6 mildly activates Stat1 in PC12 cells (32), it is not surprising to observe a 2-fold activation of luciferase activity by IL-6 in cells co-expressing FS1 (data not shown). Transcriptional specificity of Stat1 and Stat3 may be determined by cooperative binding interactions between STAT dimers and natural sequences of physiologically relevant genes (55). Stat1 may produce a weaker transcriptional activation than Stat3 on these sequences, and thus overexpression of Stat1 produces overall diminished transcriptional and neurite outgrowth responses to IL-6. Since the genes involved in the neurite outgrowth response are not yet identified, it is difficult to assess this possibility directly.

In order to further determine the specificity of Stat1 constructs, E2 cells were transiently transfected with Stat1 reporter genes, pTAL-LucGAS, containing four copies of Stat1-specific binding sites, from the murine LY-6E gene GAS element (48), together with various Stat1 constructs. Importantly, interferon-γ, a potent activator of Stat1, induced a much stronger activation of luciferase activity in cells co-expressing FS1 than in cells co-expressing a control plasmid FPDI (Fig. 5B). In contrast, expression of FS1-YF or FS1-DM inhibited the luciferase activation by interferon-γ. Unlike the Ser → Ala mutation on FS3, expression of FS1-SA produces similar effects as wild type FS1, suggesting that serine phosphorylation is not essential in the transcriptional activation by Stat1 in E2 cells.

**The Effects of Dominant Negative RAS**—IL-6-mediated dif-
The ability of IL-6 to induce neurite outgrowth in DN-RAS27 and DN-RAS12 was also examined. The dominant negative RAS produces little effect on IL-6-induced response. Neurite outgrowth by IL-6 is slightly reduced after 1 day of treatment in both DN-RAS27 and DN-RAS12 as compared with pCMV-Hygro-transfected cells. The maximal response by IL-6 in DN-RAS27 after 4 days of treatment was similar to that observed in control cells, whereas the response in DN-RAS12 is slightly reduced by 15%. These results provide direct evidence that a RAS-independent signaling mechanism is capable of inducing morphological differentiation by PC12-E2 cells.

The Effect of the Dominant Negative Form of SHC—Activation of TrkA by NGF leads to association of SHC through its phosphotyrosine-binding domain and formation of SHC-Grb2-Sos complexes, which in turn leads to activation of the RAS/ERK pathway (47). The role of SHC in IL-6-mediated response is less clear; however, it has been suggested that SHC may act as an adaptor protein for gp130-mediated responses (60, 61). SHC associates with phosphorylated gp130 through its Src homology-2 domain (SH2) domain or with activated Jak2 through its phosphotyrosine-binding domain. As with activation of TrkA by NGF, following IL-6 stimulation, formation of SHC-Grb2-Sos complexes may lead to activation of the RAS/ERK signaling pathway (61). To examine if SHC-associated signaling pathway plays a role in IL-6-induced neurite outgrowth, PC12-E2 cells were transiently transfected with dominant negative forms of SHC, the phosphotyrosine-binding domain of SHC protein (GST-N-SHC), the SH2 domain of SHC (GST-SH2), or SHC with triple mutations on tyrosine, GST-SHC-Y239F/Y240F/Y317F (GST-SHC-3YF) (47). It has been shown that overexpression of these GST-SHC mutants in PC12 cells leads to inhibition of downstream signaling pathways activated by NGF or EGF. E2 cells expressing control or mutant constructs were identified by immunostaining using an anti-GST polyclonal antiserum and FITC-conjugated second antibodies as described previously. The NGF response was reduced by about 60% at 40 h in E2 cells expressing GST-N-SHC or GST-SHC-3YF and was not significantly affected by GST-SH2 (Fig. 3). These results suggest that NGF-induced neurite outgrowth response is partly dependent on the formation of SHC-Grb2-Sos1 complexes. In contrast, the response by IL-6 is largely unaffected by all mutants, suggesting that SHC is not an important adaptor protein for gp130-mediated signaling mechanisms in PC12-E2 cells.

The Effect of MAPKK Inhibitor PD98059—RAS targets multiple effectors and activation of RAS leads to activation of multiple intracellular signaling cascades (62). Although the RAS/ERK signaling pathway is necessary for the neuronal differentiation by NGF, accumulating evidence has suggested that a RAS-dependent but ERK-independent mechanism may also be involved. A specific MAPKK inhibitor, PD98059, was used to determine the involvement of ERK in NGF and IL-6-induced neurite outgrowth. ERK activity was reduced in a dose-dependent fashion in cells pretreated with PD98059 at concentrations greater than 30 μM. Although not completely inhibited, ERK activity was markedly reduced at the maximal concentration tested. PD98059 at concentrations greater than 30 μM exhibits cytotoxicity in PC12-E2 cells. The weak activation of ERK by IL-6 was completely inhibited by treatment with PD98059. It was found that PD98059 at 30 μM reduced the NGF- and IL-6-induced neurite outgrowth response at 24 h by 20 and 30%, respectively (Fig. 7). These findings suggest that additional
ERK-independent pathways are involved in the NGF response in E2 cells and that the RAS-ERK pathway is not essential for the IL-6-mediated response.

**DISCUSSION**

Multiple signaling pathways are involved in the regulation of morphological differentiation by PC12 cells. It is well established that activation of the RAS/ERK pathway is essential for NGF- or bFGF-induced neurite proliferation, although additional signaling pathways may also be needed (63) or, under appropriate conditions, might substitute. In any case, until the essential genes that are necessary for this process are defined, it will not be possible to decide a priori which factors will be active in a positive manner and which will be inhibitory.

As with most cytokines acting on various cell types, IL-6 predominantly activates Jak/STAT pathways in PC12 cells by phosphorylation events. Tyrosine phosphorylation is required for the dimerization and translocation of Stat1 and Stat3 to the nucleus; however, the role of serine phosphorylation is less clear (56–58, 64–66). The present study demonstrates, using PC12-E2 cells transiently transfected with dominant negative forms of Stat3 proteins, that the activation of Stat3 is required for the IL-6-induced neurite outgrowth. The induction of neurite proliferation was markedly inhibited in cells overexpressing Stat3-YF or Stat3-DM, whose inhibitory functions have been well characterized (50–53). Stat3 with a Y705F mutation may be recruited to the phosphorylated tyrosine residues on the cytoplasmic tail of the gp130 through its Src homology 2 domain, but it cannot be tyrosine-phosphorylated, dimerized, or translocated to the nucleus upon IL-6 stimulation. Thus, the dominant negative effect of FS3-YF is most likely due to its competition with the endogenous Stat3 for docking sites. When introduced into cultured cells, they efficiently prevent endogenous Stat3 DNA binding and activation of target gene transcription.

IL-6, in addition to its rapid activation of Stat3 tyrosine phosphorylation, also stimulates a delayed and transient increase in serine phosphorylation (32). It has been shown that Stat3 with S727A mutation may bind DNA but with a reduced transcriptional ability (56). Overexpression of FS3-SA modestly reduces the neurite proliferation induced by IL-6. This suggests that full transcriptional activation of Stat3 in PC12 cells requires both tyrosine and serine phosphorylation, which is consistent with reports by others that IL-6-mediated response requires an H7-sensitive kinase (67). The identity of the serine kinases involved, however, is not clear. Overexpression of wild type Stat3 did not enhance the response to IL-6, indicating that the biological response to IL-6 in PC12 cells is most likely limited by the extent of activation and formation of the receptor and gp130 signaling complexes. Collectively, these results support the view that IL-6-induced neurite outgrowth is dependent on activation of the Stat3.

In PC12 and PC12-E2 cells, IL-6 activates both Stat3 and Stat1 proteins. However, it induces a more sustained and stronger stimulation of tyrosine phosphorylation of Stat3 than Stat1, which correlates with a more intense formation of SIFB and SIFC (32). In addition, the expression of Stat3 is up-regulated in IL-6-treated cells. If Stat1 and Stat3 exhibit similar biological functions in PC12 cells and a stronger activation of Stat3 is merely a reflection of the relative concentration of endogenous Stat3 to Stat1, it would be expected that...
overexpression of Stat1 would either enhance or at least have no effect on the response to IL-6. On the contrary, overexpression of Stat1 molecules leads to a partial inhibition of the neurite outgrowth by IL-6. It was found that overexpression of FS1 alters the pattern of STAT-DNA complexes. The inhibition on neurite proliferation correlates with an inhibition of the activation of endogenous Stat3 homodimers or formation of SIFA. It appears that Stat3 homodimers are the transcription factors involved in the regulation of PC12 differentiation, whereas Stat1 homodimers or Stat1-Stat3 heterodimers display a negative effect on Stat3-mediated mechanisms. These results further suggest that Stat1 and Stat3 proteins are not only differentially regulated in PC12 cells by IL-6, but also they may play opposite (or at least different) roles in the regulation of neuronal differentiation.

Similar to the dominant negative effect of FS3-YF, the mechanism by which Stat1 acts as a partial dominant negative inhibitor of Stat3 signaling pathway is apparently through competition with endogenous Stat3 for the docking sites on the cytoplasmic domain of gp130. It has been demonstrated that activation of Stat3 and Stat1 via the cytoplasmic domain of gp130 is mediated by multiple independent docking sites (68, 69). Four carboxyl-terminal tyrosine-containing motifs involving Tyr767, Tyr814, Tyr905, and Tyr915 are able to recruit Stat3. However, only two of the four tyrosine motifs surrounding Tyr905 and Tyr915 are capable of activating Stat1. This may partly explain the preferential activation of Stat3 by IL-6 and the fact that overexpression of Stat1 produces a partial inhibitory effect on Stat3 activation.

The differential role of Stat1 and Stat3 in cellular regulation has also been suggested in other cell systems. It has been reported that activation of Stat3 but not Stat1 is required for IL-6-induced terminal differentiation of myeloid leukemia M1 cells (51, 52, 70) as well as EGF receptor-mediated cell growth in transformed squamous epithelial cells (71). Although Stat1 is critical for interferon signaling and innate response to viral and bacterial infection, recent studies suggest that it also regulates caspase expression and promotes apoptosis (72–74). The specific role of Stat1 in PC12 cells, however, was not determined in the present study.

Induction of neurite outgrowth by NGF but not EGF has been attributed to the ability of NGF to induce a sustained activation of ERK (39, 75). EGF induces a short and relatively weak activation of ERK in both PC12 and PC12-E2 cells and fails to stimulate neurite proliferation in either. IL-6 is capable of activating multiple signaling pathways including RAS/ERK in many cell types; however, it has little effect on ERK in some neuronal cells including both PC12 and PC12-E2 cells (60, 61, 76–79). In fact, the effect on ERK by IL-6 is delayed and much weaker than that induced by EGF (32, 33). Thus, the activation of ERK by IL-6 alone would not be expected to produce a signal sufficient to induce neurite proliferation. Since a synergistic response was observed between IL-6 and subthreshold concentrations of NGF or EGF, it is possible that weak activation of the RAS/ERK pathway may contribute in some way to the overall response. Data presented in the present study, however, do not identify a significant contribution of RAS/ERK pathway to the induction of neurite outgrowth by IL-6. IL-6 is capable of inducing robust neurite outgrowth in PC12-E2 cells expressing dominant negative RAS or SHC entities. The MAPKK inhibitor, PD98059, did produce a slight reduction in the percentage of responsive cells, so the weak activation of Fig. 7. The effect of MAPKK inhibitor, PD98059, on protein tyrosine phosphorylation of ERK and neurite outgrowth induced by NGF and IL-6 in PC12-E2 cells. A, PC12-E2 cells were grown in complete medium for 2 days and continued in culture in low serum medium for 1 day before the treatment. Cells were pretreated with various concentrations of PD98059 for 1 h before stimulation with 100 ng/ml NGF for 5 min or 30 ng/ml IL-6 for 15 min. Immunoblotting conditions were the same as described in the legend to Fig. 5A. B, to determine the effect of PD98059 on neurite outgrowth, cells were grown in complete medium for 16 h, changed to low serum medium, and pretreated with PD98059 for 1 h before the addition of 30 ng/ml IL-6 (solid circle), 100 ng/ml NGF (solid triangle), or no factors (open circle) for 24 h. The percentage of responsive cells was scored. Vertical bars represent S.E. (n = 3).
ERK may contribute to cell cycle arrest but is not likely to be important for the IL-6-induced neurite proliferation.

The results presented herein are in contrast to the findings of Ihara et al. (35). These workers utilized PC12 cells microinjected with chimeric receptors consisting of the extracellular domain of the granulocyte colony-stimulating factor receptor and the cytoplasmic domain of gp130, including various mutations and truncations. The cells were pretreated with NGF for 1–2 h and then stimulated with granulocyte colony-stimulating factor to activate the chimera. They observed that elimination of the STAT binding sites on gp130 actually stimulated differentiation and concluded that IL-6 also induced neurite outgrowth via ERK activation (as with NGF). The Stat3 activation was deemed to be inhibitory, and they proposed that the brief exposure of the cell to NGF suppressed the Jak/STAT pathway, allowing the ERK pathway to then be manifested. Because of the nature of the experimental system (microinjection of single cells), these studies did not allow any direct evaluation of the pathways stimulated, and the specificity of the chimeras was dependent on evaluation in other cell types (70). Under the conditions of their experiments, ERK activation may indeed become the dominant pathway in PC12 cells (80). However, clearly Stat3 activation is not inherently inhibitory and can, at least in PC12-E2 cells, lead directly to differentiation. It should be noted that Ihara et al. (35) studied native PC12 cells, while the studies reported herein were done with PC12-E2 cells, and undefined differences between them may underlie the differences in response observed.

The nature of the PC12 cells used in signaling experiments is also clearly important. IL-6-induced differentiation of PC12 cells is dependent on other treatments or conditions than are found in native PC12 cells (not previously exposed to stimuli). Primed cells or PC12-E2 cells give robust, transcription-dependent responses to IL-6. More transient exposure to NGF (1–2 h) (35) can also produce a responsive condition; however, we observed that only one of three PC12 cell lines in our laboratory showed this response (data not shown). Thus, this “initiation” phenomenon requires certain cellular conditions (in addition to the NGF-induced effects) to achieve IL-6 responsiveness. We have previously suggested that priming by NGF leads to cell cycle arrest through the production of one or more suppressor proteins. These, or similar functional entities, are presumed to already be expressed in PC12-E2 cells. The response to IL-6 is dependent on these entities (and IL-6 cannot induce their synthesis directly). This is supported by the observation that primed cells remain responsive to IL-6 only transiently (~3 days), while PC12-E2 cells are permanently responsive. These observations clearly indicate that a pathway other than ERK is required for the full IL-6 response and is entirely consistent with the findings reported herein that that pathway is contributed by Stat3 activation.

Regulation of neuronal functions involves multiple signaling pathways. Activation of either RAS/ERK by polypeptide growth factors such as NGF or bFGF or activation of Jak/STAT pathway by cytokines such as IL-6 or ciliary neurotrophic factor leads to trophic, survival, or differentiative effects in various cultured cells. Thus, activation of these two pathways may lead to opposing physiological responses (17, 70). The data from the present study suggest that, in an appropriate cellular environment, stimulation of these two parallel signaling pathways by a distinct class of extracellular signaling polypeptides may act collaboratively in the regulation of neuronal differentiation.

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