Mechanisms of Signal Transduction:

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STAT3 as a Downstream Mediator of Trk Signaling and Functions*

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Signal transducer and activator of transcription 3 (STAT3) has long been shown to regulate gene transcription in response to cytokines and growth factors. Recent evidence suggests that STAT3 activation may also occur downstream of receptor-tyrosine kinase activation. In the current study we have identified STAT3 as a novel signal transducer for TrkA, the receptor-tyrosine kinase that mediates the functions of nerve growth factor (NGF). Activation of TrkA by NGF triggered STAT3 phosphorylation at Ser-727, and enhanced the DNA binding and transcriptional activities of STAT3. More importantly, neurotrophin-induced increase in STAT3 activation was observed to underlie several downstream functions of neurotrophin signaling. First of all, knockdown of STAT3 expression using the RNA interference approach attenuated NGF-induced transcription of immediate early genes in PC12 cells. Furthermore, reduced STAT3 expression in PC12 cells suppressed NGF-induced cyclin D1 expression, thereby inhibiting growth arrest normally triggered by NGF treatment. Finally, inhibition of STAT3 expression decreased brain-derived neurotrophic factor-promoted neurite outgrowth in primary hippocampal neurons. Together, our findings have identified STAT3 as an essential component of neurotrophin signaling and functions.

Neurotrophins are a family of trophic factors critical for the survival and development of neurons within the peripheral and central nervous systems. Members of the family include the prototypic member nerve growth factor (NGF),[3] brain-derived neurotrophic factor (BDNF), neurotrophin-3 (NT-3) and NT-4/5 in the mammals, and NT-6/7 in the fish species (1–4). The actions of neurotrophins are mediated by Trks, a family of receptor tyrosine kinases (RTKs). Binding of neurotrophins to their cognate receptors TrkA, TrkB, or TrKC induces rapid tyrosine phosphorylation of the receptors, resulting in their transactivation. Phosphorylated tyrosine residues on the receptors then serve as docking sites for various adaptor molecules, thereby activating multiple intracellular signal transduction pathways including Ras-mitogen-activated protein kinase, phosphatidylinositol 3-kinase, and phospholipase Cγ. Although the intricate signaling cascade activated may directly take part in mediating the downstream functions of neurotrophins, initiation of gene transcription also plays an important role (3–5). Among the transcription factors triggered by Trk signaling, for example, phosphorylation of CREB (cAMP-response element-binding protein) is involved in neurotrophin-mediated neuronal differentiation and survival (6–8). Activation of Egr-1 has been demonstrated to play crucial roles in the initiation of neurite outgrowth in PC12 cells (9). Induction of AP-1 activity, on the other hand, is implicated in the pro-survival property of NGF in serum-deprived PC12 cells (10). It is therefore of interest and importance to further elucidate the roles of various transcription factors in neurotrophin/Trk signaling.

Recently, activation of STAT3, a member of the signal transducer and activator of transcription family, has been observed after activation of several RTKs such as ErbB and Eph receptors (11–13). The identification of STAT3 in neuregulin/ErbB (12) and ephrin/Eph signaling (13) in our laboratory prompted us to investigate if STAT3 activation also plays a role in neurotrophin/Trk signaling. STAT3 was originally identified as a crucial downstream component in cytokine signaling (13–15). The canonical STAT3 activation pathway involves recruitment of STAT3 to the cytokine receptors, such as gp130 and leukemia inhibitory factor receptor, upon cytokine stimulation. The phosphorylation of STAT3 by receptor-associated Janus kinases at tyrosine 705 (Tyr-705) subsequently leads to activation and homo- or heterodimerization of the STAT1/3 transcription factors. These homo- or heterodimers of STAT1/3 then translocate to the nucleus, activating multiple target gene transcription via interaction with specific DNA-response elements (14).

In addition to Tyr-705 phosphorylation, phosphorylation of STAT3 at serine 727 (Ser-727) has also been demonstrated to play a regulatory role in STAT3 activation. The precise function of Ser-727 phosphorylation nonetheless remains obscure. It is generally believed that Ser-727 phosphorylation is required for STAT3 to achieve its maximal transcriptional activity (16, 17). However, Ser-727-phosphorylated STAT3 has also been suggested to mediate transcription activation without a detectable Tyr-705 phosphorylation (18–20). On the other hand, studies demonstrating an inhibitory effect of Ser-727 phosphorylation on Tyr-705 phosphorylation of STAT3 have been reported (19, 21, 22). The apparently contradictory findings are likely due to the use of different cellular systems and treatment paradigms, as different kinases are implicated to mediate STAT3 Ser-727 phosphorylation in different experimental systems (12, 19, 23–29).

Several studies have investigated the functional roles of STAT3 in neural stem cell differentiation, survival, and inflammatory response of neurons after injury and regulation of leptin signaling in the hypothalamus (30–32). Nonetheless, information on the functions of STAT3 in developing neurons remains limited. In this study we present evidence that STAT3 is activated upon NGF stimulation through TrkA receptor in PC12 cells. NGF treatment resulted in the induction of DNA binding

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[3] The abbreviations used are: NGF, nerve growth factor; BDNF, brain-derived neurotrophic factor; Cdk5, cyclin-dependent kinase 5; Erk, extracellular-regulated kinase; Ros, roscovitine; RTK, receptor tyrosine kinase; STAT, signal transducer and activator of transcription; MEK, mitogen-activated protein kinase/extracellular signal-regulated kinase kinase; siRNA, small interfering RNA; DIV, days *in vitro*; SIE, Sis-inducible element.

and transcriptional activities of STAT3, suggesting that activation of STAT3 might partially contribute to the transcription of NGF-induced genes. Moreover, inhibition of STAT3 expression by RNA interference attenuated the antiproliferative effect of NGF in PC12 cells via the suppression of cyclin D1 expression. Finally, reduction of STAT3 expression also markedly attenuated the promoting effect of BDNF on neurite extension in cultured hippocampal neurons. These findings reveal an essential role of STAT3 in the downstream functions of neurotrophin signaling and provide novel insights on the roles of STAT3 in the nervous system.

EXPERIMENTAL PROCEDURES

Antibodies, DNA Constructs, and siRNAs—The antibodies against phosphorylated forms of STAT3 and threonine/tyrosine-phosphorylated Erk1/2 were purchased from Cell Signaling Technology. Polyclonal antibodies that recognize Cdk5, cyclin D1, Egr-1, and p300 were purchased from Santa Cruz Biotechnology, and antibody specific for actin was from Sigma. Antibody that recognized STAT3 was from Transduction Laboratories.

The expression vectors for STAT3, STAT3M (Ser-727 to Ala-727 (12)) were provided by Dr. Zilong Wen (Institute of Molecular and Cell Biology, Singapore). The luciferase construct that was linked to the STAT3-responsive enhancer (pSTAT3-Luc) was a kind gift from Prof. Yung-hou Wong (The Hong Kong University of Science and Technology).

Stealth^{Tm} RNA interference molecules for STAT3 and Cdk5 were designed with the on-line software from Invitrogen. The sequences used were: STAT3 siRNA, GGAAAUUUAACAUUCUGGGCACGAA; control siRNA (STAT3), GGAUUUCAAUUAGUCCGGCAAAGAA; Cdk5 siRNA, CCUCCGGGAGAUCUGUCUACUCAAA; control siRNA (Cdk5), CCUAGGGCUAGCUGUUCAUCCCAAA.

Cell Cultures—PC12 cells were cultured and maintained as described (33). In brief, PC12 cells were cultured in Dulbecco's modified Eagle's medium supplemented with 6% heat-inactivated horse serum, 6% heat-inactivated fetal bovine serum, penicillin (50 units/ml), and streptomycin (100 μg/ml, Invitrogen). Cells were routinely grown on 100-mm tissue culture dishes (Falcon) at 37 °C in a humidified atmosphere with 7.5% CO_2, and medium was changed every 3 days. For NGF-induced differentiation, the cells were cultured in medium supplemented with 1% serum and 50 ng/ml NGF (Alomone Labs). For analysis of protein phosphorylation, cells were maintained in Dulbecco's modified Eagle's medium without serum for 4 h and then stimulated with NGF or BDNF for the times indicated. For inhibitor studies, cells were pretreated with K252a (100 nM), U0126 (10 μM), H7 (100 μM), roscovitine (Ros, 25 μM), or wortmannin (100 nM) for 1 h before NGF treatment. Transient transfection of STAT3 or STAT3 mutant constructs was carried out using Lipofectamine PLUS (Invitrogen) reagents according to the manufacturer's instructions. Transfection of Stealth^{Tm} RNA interference molecules was performed with Lipofectamine 2000 (Invitrogen) reagent as suggested by the manufacturer.

Primary cortical neuron cultures were prepared from embryonic day 18 (E18) rat embryos. Cortices were dissected in Dulbecco's modified Eagle's medium, dissociated in the same medium, and plated on poly-D-lysine-coated culture plates. Cells were cultured in neurobasal medium containing B27 supplement, 0.5 mM glutamine, penicillin (50 units/ml), and streptomycin (100 μg/ml). Cultures were incubated at 37 °C in a humidified atmosphere with 5% CO_2. In the study of STAT3 phosphorylation, cultures at DIV7 (7 days *in vitro*) were washed with neurobasal medium twice and kept in the same medium for 1 h. BDNF at 50 ng/ml was added for the time indicated. Primary hippocampal neurons were prepared from E18 embryos and cultured using similar procedures as described above for cortical neurons. Transfection of Stealth^{Tm} RNA interference and green fluorescent protein-expressing vector was performed on culture at DIV2 with Lipofectamine 2000 reagent as described by the manufacturer. BDNF at 50 ng/ml was added for 2 days after the transfection.

Immunohistochemical Analysis—After fixation with 4% paraformaldehyde for 30 min at room temperature, PC12 cells were blocked with 4% goat serum in phosphate-buffered saline containing 0.4% Triton X-100 for 20 min at room temperature. Cells were then incubated with STAT3 polyclonal antibody (Santa Cruz, 1:1000), anti-phosphotyrosine STAT3 antibody (Cell Signaling, 1:1000), or anti-phosphoserine STAT3 antibody (Cell Signaling, 1:1000) at 4 °C overnight and washed 3 times with phosphate-buffered saline. Fluorescein isothiocyanate-conjugated anti-mouse or anti-rabbit (Amersham Biosciences, 1:1000) was added and incubated for 1 h at room temperature. After extensive washing with phosphate-buffered saline, cells were mounted with Mowiol and analyzed under Olympus confocal microscope.

Western Blot Analysis and Immunoprecipitation—PC12 cells were harvested and lysed in modified radioimmune precipitation assay lysis buffer with 150 mM NaCl, 1% Nonidet P-40, 1 mM EDTA, 0.5% deoxycholic acid, 2 μg/ml aprotinin, 1 mM phenylmethylsulfonyl fluoride, 5 mM benzamidine, 1 mM sodium orthovanadate, and 10 μg/ml soybean trypsin inhibitor (Sigma) in 50 mM Tris buffer, pH 7.4. The proteins separated by SDS-PAGE gel electrophoresis were then transferred onto a nitrocellulose membrane (Micron). After blocking with 0.1% Tween 20 and 5% nonfat dry milk in Tris-buffered saline at room temperature for 1 h, the membrane was incubated with primary antibody (1:1000) at 4 °C overnight and horseradish peroxidase-conjugated secondary antibody (1:2000) for 1 h and detected using the Enhanced Chemiluminescence (ECL) Western blot System (Amersham Biosciences).

For immunoprecipitation, cell lysates (1 mg) were incubated with 1 μg of indicated antibody at 4 °C overnight followed by the addition of 20 μl of protein G- Sepharose for 1 h at 4 °C. After extensive washing with the lysis buffer, immunoprecipitates were resolved by SDS-PAGE gel electrophoresis as described above. Immunoblots were scanned, and the band intensity was measured using ImageJ software (National Institutes of Health). Quantification was performed with results from three separate experiments.

DNA Binding Assay—Total cellular extract of PC12 cells was prepared using the lysis buffer (15 mM HEPES, pH 7.6, 40 mM KCl, 1 mM EDTA, 1 mM dithiothreitol, 5% glycerol) as previously described (12) and incubated with 10 μg of SIE oligo-agarose beads (Santa Cruz) for 30 min at room temperature. After incubation, the beads were washed extensively with the lysis buffer. The SIE bead-precipitated proteins were separated by SDS-PAGE and then transferred to nitrocellulose membrane for immunoblotting with monoclonal antibody against STAT3.

Promoter Luciferase Assay—PC12 cells were seeded at a density of 2×10^5 per well in 24-well plates. Twenty-four hours after seeding, cells were transfected with the corresponding reporter construct and STAT3, STAT3M, or empty vector in a ratio of 1:9. β-Galactosidase-pCMV construct (25 ng, Clontech) was included in the transfection mix for normalization. Luciferase assay was performed 1 day after the transfection using a kit purchased from Promega, and β-galactosidase activity was measured using luminescent β-galactosidase enzyme kit (BD Biosciences). Luciferase activity was normalized against β-galactosidase activity to correct for the variation in transfection efficiency. For cells transfected with siRNA, siRNA transfection was performed after 1 day of DNA transfection of the reporter constructs. The experiments were

performed in triplicate, and similar results were obtained for at least three independent experiments.

Reverse Transcription-PCR, Southern and Northern Blot Analyses—Total RNA of PC12 cells was extracted using acid guanidinium thiocyanate method as previously described (34). Total RNA was analyzed by formaldehyde RNA gel, and Northern hybridization against cDNA probes corresponding to partial sequence of *egr-1*, *junB*, or *gapdh* was performed as described previously. For the synthesis of cDNA, 5 μg of total RNA was mixed with 0.5 μM oligo-dT primer (Invitrogen) and denatured at 70 °C for 10 min. After cooling on ice, reverse transcription mixture (1× PCR buffer, 2.5 mM $MgCl_2$, 0.5 mM dNTPs, 10 mM dithiothreitol, and 10 units/ml Superscript II reverse transcriptase) was added. The reaction was carried out at 42 °C for 50 min and terminated at 70 °C for 15 min. One-tenth of the cDNA mixture was used as the template for the subsequent PCR amplification. PCR primers were: *cyclin D1*, 5′-primer, 5′-ATGGAACACCAGCTCCTGTGC-3′, and 3′-primer, 5′-ATGTCCACATCTCGGA-3′; *stat3*, 5′ primer, 5′-ATTGACCTGCCGATGTCCCCCCGCACTTTAGATTCA-3′, and 3′ primer, 5′-TCACATCGGGGAGGTAGC-3′; *gapdh*, 5′ primer, 5′-TGATGCTGGTGCTGAGTATGTCGTG-3′, and 3′ primer, 5′-TCCTTGGAGGCCATGTAGGCCAT-3′. PCR products were separated on 1% agarose gel and then transferred onto nylon membranes. DNA blots were hybridized with appropriate probes and exposed to Eastman Kodak Co. XAR-5 x-ray film at −80 °C overnight.

Cell Proliferation Assay for Transfected PC12 Cells—PC12 cells were seeded at a density of 1×10^5 per well in 24-well plates. Twenty-four hours after seeding, cells were transfected with the control or STAT3 siRNAs. Cells were treated with NGF for the times indicated. Growth of the transfected PC12 cells were measured by counting the number of cells using a hemocytometer.

The 5-bromo-2′-deoxyuridine labeling method was performed to examine cell proliferation by measuring DNA replication in NGF-treated PC12 cells with or without STAT3 siRNA transfection. Cells were seeded and transfected as described. The transfected cells were treated with NGF for 4 days and then labeled with 5-bromo-2′-deoxyuridine for 3 h. Cells were then fixed and stained as described (35).

Quantitation of Neurite Extension in Hippocampal Neurons—Morphological analysis of transfected hippocampal neurons (DIV4) was performed using the images acquired with phase-contrast microscope (Zeiss). The length of the longest neurite was traced using MetaMorph Version 5.0r1 software (Universal Imaging Corp.). For each measurement at least 50 cells per slide were counted from randomly selected fields and $n = 3$ slides. Each experiment was repeated three times.

Statistical Analysis—Results from the morphological studies and luciferase assays were analyzed by a two-tailed Student's *t* test. A *p* value of less than 0.05 was considered to be statistically significant.

RESULTS

Phosphorylation of STAT3 Was Induced by Neurotrophins in Neuronal Cells—PC12, a rat adrenal pheochromocytoma cell line, has been widely used as a model system for examining NGF-induced differentiation of neuronal cells due to its expression of TrkA and the well characterized downstream signaling triggered by NGF treatment. We have, therefore, adopted this cell line to examine the activation of STAT3 in response to NGF stimulation and the functional significance of STAT3 activation. In agreement with previous findings, treatment of PC12 cells with NGF induced rapid Erk1/2 activation (Fig. 1*A* (36)). Interestingly, we found that NGF also induced Ser-727 phosphorylation of STAT3 within 5 min. The levels of Ser(P) STAT3 remained elevated up to 30 min of NGF stimulation (Fig. 1*A*). It is noteworthy that Tyr-705-phosphorylated STAT3 was barely detected in PC12 cells before and after NGF stimulation (Fig. 1*A*).

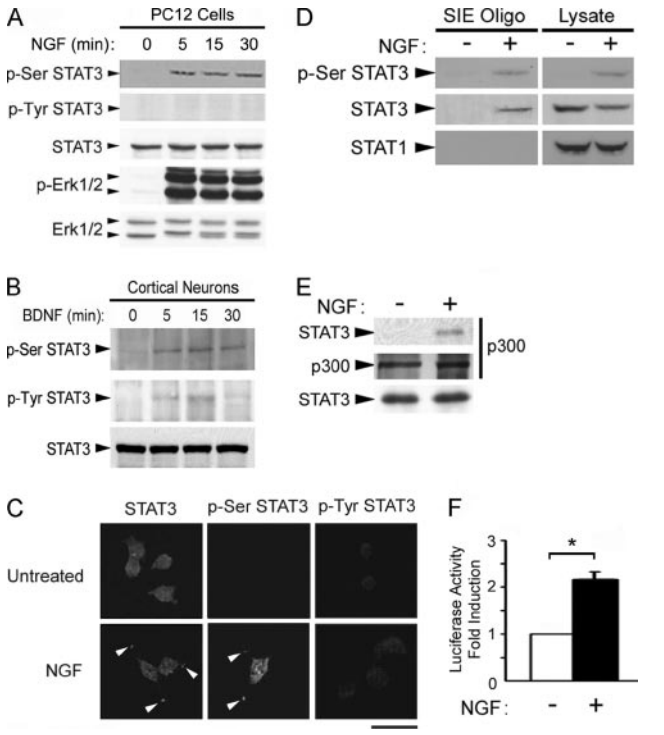

FIGURE 1. **Phosphorylation of STAT3 proteins was induced by neurotrophins in PC12 cells and cultured cortical neurons.** *A*, naïve PC12 cells were serum-starved for 4 h and then treated with NGF (50 ng/ml) for 5 min to 30 min. Total cell lysates were subjected to Western blot analysis using antibodies specific for Ser(P) (*p-Ser*) STAT3, Tyr(P) (*p-Tyr*) STAT3, phosphorylated (*p-*)Erk1/2, STAT3, and Erk1/2. *B*, cortical neuron cultures (DIV 7) were treated with BDNF (50 ng/ml) for 5–30 min, and the total cell lysates were analyzed with the indicated antibodies. *C*, after stimulation with NGF for 10 min, cells were fixed and stained with STAT3, Ser(P) STAT3, and Tyr(P) STAT3. *Arrowheads* indicate the growth cones. *Scale bar*, 50 μm. *D*, after serum starvation for 4 h, PC12 cells were treated with NGF for 15 min. Total lysates were then incubated with SIE-agarose beads. Pulled-down STAT3 was detected by Western blotting using specific antibodies against Ser(P) STAT3, STAT3, and STAT1. *E*, anti-p300 antibody was used to immunoprecipitate p300 from PC12 cell lysates after treatment with NGF for 15 min. The immunoprecipitated complexes were resolved and analyzed with p300 and STAT3 antibodies. A Western blot of STAT3 from whole cell lysates was shown as the loading control. *F*, PC12 cells were transfected with pSTAT3-Luc reporter. At 24 h post-transfection, the transfected cells were serum-starved for 4 h and then treated with NGF for 1 day. Cells were subjected to luciferase assay. Results were normalized with β-galactosidase activity; mean ± S.E., $n = 3$; *, $p < 0.05$.

To determine whether neurotrophin treatment also induced phosphorylation of STAT3 in neurons, primary cortical neurons (DIV7) were treated with BDNF, and phosphorylation of STAT3 was also observed (Fig. 1*B*). Application of BDNF to cortical neurons for 5 min triggered phosphorylation of STAT3 at Ser-727 and Tyr-705. The level of Tyr(P) STAT3 declined after 15 min of BDNF treatment, but Ser(P) STAT3 remained elevated. Taken together, these results indicate that neurotrophins induced phosphorylation of STAT3 in neuronal cells. The induction of Tyr-705 phosphorylation of STAT3 in cortical neurons may reflect the possibility that different regulatory mechanisms for STAT3 phosphorylation are involved in PC12 cells and in primary neurons.

We next examined the subcellular localization of STAT3 and its phosphorylated forms after NGF treatment using immunocytochemistry. In untreated PC12 cells, STAT3 was located at both the cytoplasm and nuclei. Treatment with NGF for 10 min induced the translocation of STAT3 to the growth cones (Fig. 1*C*). Using the phospho-specific antibodies of STAT3, we found that before NGF treatment, Ser(P)

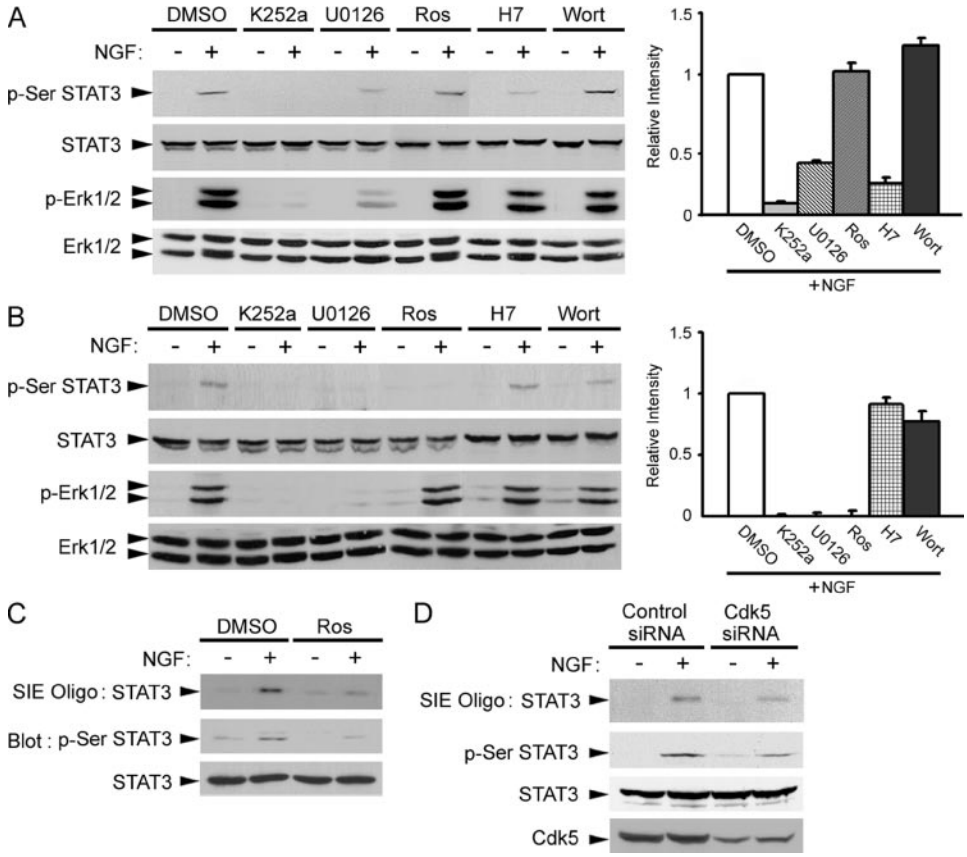

FIGURE 2. **Ser-727 phosphorylation of STAT3 by NGF occurred through several signaling pathways.** K252a (100 nM), U0126 (10 μM), Ros (25 μM), H7 (100 μM), and wortmannin (*Wort*, 100 nM) were used to pretreat naïve (*A*) and 1-day-NGF-primed PC12 cells for 1 h (*B*). Cells were then stimulated with NGF for 15 min. Total cell lysates were subjected to Western blot analysis using antibodies specific for Ser(P) (*p-Ser*) STAT3, phosphorylated (*p-*) Erk1/2, STAT3, and Erk1/2. Quantitation shown in *A* and *B* was the relative levels of Ser(P) STAT3 detected as compared with the respective Me_2SO (*DMSO*)-treated samples; mean $\pm$ S.E., $n = 3$. *C*, PC12 cells were NGF-primed for 1 day. After serum starvation for 4 h and pretreatment with Ros (25 μM) for 1 h cells were treated with NGF in the presence or absence of Ros for 5 min. STAT3 DNA binding was determined as in Fig. 1*C*. *D*, PC12 cells were transfected with Cdk5 siRNA and primed with NGF for 1 day. After serum starvation for 4 h cells were treated with NGF for 5 min. SIE pull-down assay was done as before.

STAT3 and Tyr(P) STAT3 expression was undetectable. Upon NGF stimulation, Ser(P) STAT3 was localized to the cytoplasm, nuclei, and growth cones of PC12 neurites, but Tyr(P) STAT3 remained below detectable level (Fig. 1*C*, *right panel*). Our data, therefore, verified that in PC12 cells NGF treatment mainly resulted in Ser-727 phosphorylation of STAT3.

NGF Stimulation Induced Increases in DNA Binding and Transcriptional Activity of STAT3—Because the relative importance of Ser-727 and Tyr-705 phosphorylation of STAT3 on its transcriptional activity appears to vary depending on the cellular context, it is crucial for us to examine if the induction of Ser-727 phosphorylation of STAT3 by NGF affects STAT3 activity. We first examined if NGF treatment modulated the DNA binding activity of STAT3 using SIE oligo-agarose beads. We found that NGF induced STAT3 DNA binding in PC12 cells, which was not observed in the absence of NGF stimulation (Fig. 1*D*). When we stripped the blot and re-probed with phospho-specific STAT3 antibodies, only Ser(P) STAT3 (Fig. 1*D*), but not Tyr(P) STAT3 (data not shown), was detected. Because STAT3 can form heterodimer with STAT1, we used a STAT1-specific antibody to examine if the association between STAT3 and SIE oligo involved STAT1/STAT3 heterodimer. STAT1 could not be detected in the SIE oligo-agarose bead precipitated complex (Fig. 1*D*), suggesting that NGF stimulation mainly led to STAT3 homodimerization.

Acetylation of STAT3 at Lys-685 by p300 has recently been reported to be critical for cytokine-mediated dimerization, DNA binding, and transcriptional activity of STAT3 (37, 38). Because Ser-727 of STAT3 is suggested to play a crucial role in the association of STAT3 with p300 upon cytokine stimulation (17, 37–39), the induction of STAT3 Ser-727 phosphorylation by NGF might affect the association of p300 with STAT3, thereby modulating its transcriptional activity. Consistent with this notion, we found that STAT3 associated with p300 after 15 min of NGF treatment, whereas no interaction between these two molecules was observed in the absence of NGF (Fig. 1*E*). Our data, therefore, demonstrated that not only did NGF treatment induce phosphorylation of STAT3, but it also facilitated association of STAT3 with p300, enabling potential acetylation and activation of STAT3 in PC12 cells.

The association of STAT3 with SIE oligo and p300 together with the induction of Ser-727 phosphorylation only in the presence of NGF indicates that NGF treatment initiated STAT3 transcription in PC12 cells. To further characterize if NGF also affects STAT3 transcriptional activity, PC12 cells were transfected with luciferase construct linked to the STAT3-response enhancer element (pSTAT3-Luc). Corroborating with the NGF-induced binding of STAT3 to SIE oligo, NGF treatment induced the promoter activity of STAT3 (Fig. 1*F*). Taken together, our data demonstrated that NGF could induce both STAT3 DNA binding and transcriptional activities in PC12 cells.

Phosphorylation of STAT3 at Ser-727 Was Mediated by Distinct Signaling Pathways—Having verified that NGF induced the serine 727 phosphorylation of STAT3 in PC12 cells, we proceeded to characterize the signaling pathway(s) involved in the Ser-727 phosphorylation of STAT3 using a panel of selective inhibitors. TrkA inhibitor K252a (100 nM), MEK1/2 inhibitor U0126 (10 μM), phosphatidylinositol 3-kinase inhibitor wortmannin (100 nM), serine/threonine kinase inhibitor H7 (100 μM), and Cdk5 inhibitor Ros (25 μM) were used to pretreat PC12 cells for 1 h before 15 min of NGF treatment. As previously demonstrated, pretreatment of PC12 cells with selective Trk inhibitor K252a abolished NGF-induced STAT3 phosphorylation at Ser-727, suggesting that the induction of STAT3 phosphorylation occurred downstream of TrkA activation. Interestingly, we found that different signaling pathways were implicated in STAT3 activation in naïve or NGF-primed PC12 cells after transient NGF treatment. In naïve PC12 cells, U0126 and H7 attenuated Ser(P) STAT3 phosphorylation (Fig. 2*A*), indicating

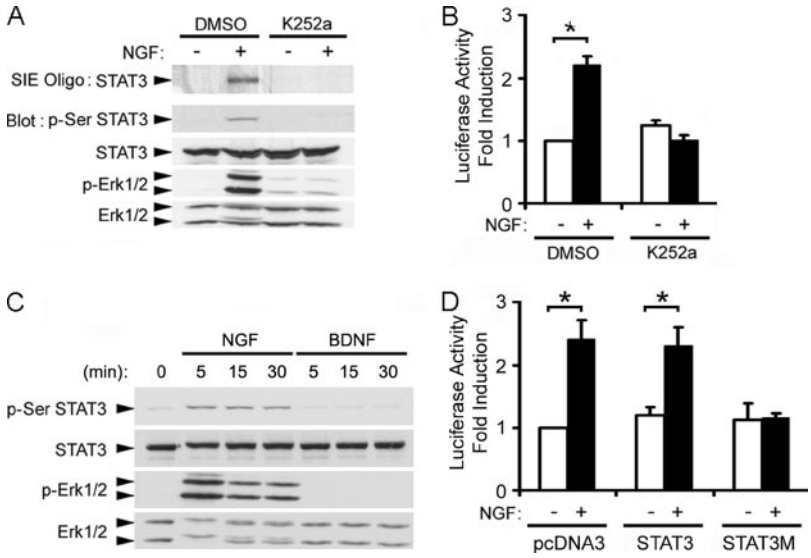

FIGURE 3. **NGF induced transcriptional activity of STAT3 in PC12 cells through TrkA activation and Ser(P) (*p-Ser*) STAT3.** *A*, PC12 cells were pretreated with K252a and then treated with NGF for 15 min. Total lysates were then incubated with SIE-agarose beads. Pulled-down STAT3 was detected by Western blotting using specific antibody. *p-*, phosphorylated. *DMSO*, Me_2SO. *B*, PC12 cells were transfected with pSTAT3-Luc reporter. At 24 h post-transfection, the transfected cells were serum-starved for 4 h and pretreated with K252a. Cells were treated with NGF for 1 day and then subjected to luciferase assay. *C*, naïve PC12 cells were serum-starved for 4 h and then treated with NGF or BDNF (50 ng/ml) for 5–30 min. Total cell lysates were subjected to Western blot analysis using antibodies specific for Ser(P) STAT3, phosphorylated-Erk 1/2, STAT3, and Erk1/2. *D*, PC12 cells were transfected with pSTAT3-Luc reporter together with STAT3 or STAT3M. At 24 h post-transfection, the transfected cells were serum-starved for 4 h and stimulated with NGF for 1 day and subsequently lysed for luciferase assay. Results were normalized with β-galactosidase activity; mean ± S.E., $n = 3$; *, $p < 0.05$.

that Ser-727 phosphorylation of STAT3 was mediated by the MEK/Erk pathway and an H7-sensitive pathway. On the other hand, Ser-727 phosphorylation of STAT3 was blocked by U0126 and Ros, and slightly reduced by wortmannin treatment in PC12 cells primed with NGF for 1 day (Fig. 2*B*), suggesting the involvement of MEK/Erk pathway, phosphatidylinositol 3-kinase signaling, and Cdk5 activity.

As shown in Fig. 2*B*, NGF-induced Ser-727 phosphorylation of STAT3 in 1-day-NGF-primed PC12 cells required at least in part Cdk5 activity. Our observations corroborated and extended the findings of several previous reports (40–42). The serine/threonine kinase Cdk5 is activated upon association with its activator p35 or p39. PC12 cells exhibit increased p35 protein levels and Cdk5 activity upon differentiation with NGF treatment (41). The induction of p35 expression in PC12 cells after 1 h of NGF treatment suggests that the normally quiescent Cdk5 can be activated by NGF in these cells (40, 41). Our laboratory has previously demonstrated that Ser-727 of STAT3 serves as a potential phosphorylation site for the active Cdk5 complex (12). We are thus interested in further exploring if Cdk5 is implicated in maintaining the transcriptional activity of STAT3 after 1 day of NGF treatment. The DNA binding ability of STAT3 to SIE oligonucleotide was examined in NGF-primed PC12 cells in the presence or absence of Cdk5 inhibitor Ros. We found that Ros pretreatment markedly reduced NGF-triggered STAT3 DNA binding activity in addition to suppressing Ser-727 phosphorylation of STAT3 (Fig. 2*C*). Inhibition of Cdk5 activity using Cdk5 siRNA (Fig. 2*D*) also attenuated NGF-induced Ser(P) STAT3 levels and STAT3 DNA binding activity. Taken together, our findings identified Cdk5 as a downstream signal modulator for serine phosphorylation of STAT3 in response to 1 day of treatment with NGF.

NGF-induced STAT3 Transcriptional Activity Required TrkA Activation and Ser-727 Phosphorylation of STAT3 in PC12 Cells—In addition to Trk receptors, neurotrophins can also initiate downstream signaling via activation of the low affinity neurotrophin receptor, p75. Because PC12 cells express both TrkA and p75 (43), induction of STAT3 activation by NGF can occur via TrkA, p75, or both. We were thus interested in examining if NGF-induced phosphorylation of STAT3 was mediated by TrkA activation. Importantly, abrogation of Ser(P) STAT3 by K252a pretreatment (Fig. 3*A*) was accompanied by a marked reduction in the DNA binding activity and transcriptional activity of STAT3 (Figs. 3, *A* and *B*), suggesting that TrkA activation is essential for NGF-induced STAT3 activation. To further verify if STAT3 activation involved p75, PC12 cells were treated with BDNF. Because PC12 cells express no TrkB or TrkC, BDNF stimulation would lead to activation of p75 alone, thus allowing us to examine if neurotrophin-triggered STAT3 activation involves p75. Interestingly, in contrast to a clear induction of Ser(P) STAT3 and phosphorylated-Erk 1/2 in the presence of NGF, no phosphorylation of STAT3 and Erk1/2 was detected after BDNF stimulation (Fig. 3*C*), suggesting that activation of p75 alone could not activate STAT3. These observations collectively indicate that NGF-induced serine phosphorylation and activation of STAT3 both required TrkA activation.

Because Ser-727 phosphorylation of STAT3 has been implicated to regulate transcriptional activity of STAT3 and was consistently observed after NGF treatment, we were interested in examining if NGF-induced Ser-727 phosphorylation of STAT3 was required for the activation of STAT3 transcriptional activity. A Ser-727 STAT3 mutant (serine to alanine; STAT3M) was generated. When STAT3M was co-transfected with the reporter construct, the NGF-induced STAT3 promoter activity was abolished (Fig. 3*D*). This observation thus revealed that NGF-induced STAT3 transcriptional activity in PC12 cells occurred via phosphorylation of STAT3 at Ser-727 residue.

Activation of STAT3 Contributed to NGF-induced Immediate Early Gene Transcription in Naïve PC12 Cells—The induction of STAT3 activation by NGF reported here might constitute one of the mechanisms by which NGF triggers gene transcription. We therefore examined the effect of STAT3 inhibition on NGF-induced gene transcription. When STAT3 expression was reduced by STAT3 siRNA in PC12 cells, the level of NGF-mediated Ser(P) STAT3 was diminished (Fig. 4*A*). This was accompanied by the inhibition of STAT3 DNA binding and promoter activities in STAT3 siRNA-transfected cells (Figs. 4, *A* and *B*). We further examined if NGF-induced genes were also affected by STAT3 inhibition. Among the genes induced by NGF treatment, *egr-1* and *junB* are of particular interest because their transcription has previously been demonstrated to involve STAT3 after stimulation by other trophic factors such as leukemia inhibitory factor and neuregulin (12, 44). Here we found that in PC12 cells transfected with STAT3 siRNA, NGF-induced expression of *egr-1* and *junB* mRNAs was inhibited in the Northern blot analysis (Fig. 4*C*), indicating that NGF-triggered transcription of *egr-1* and *junB* both involved STAT3 activation. In addition, NGF-induced elevation of Egr-1 protein expression was also attenuated after STAT3 inhibition by STAT3 siRNA (Fig. 4*D*). Taken together, our findings indicate that NGF-induced STAT3 activation plays a functional role by at least partially mediating the NGF-triggered expression of immediate

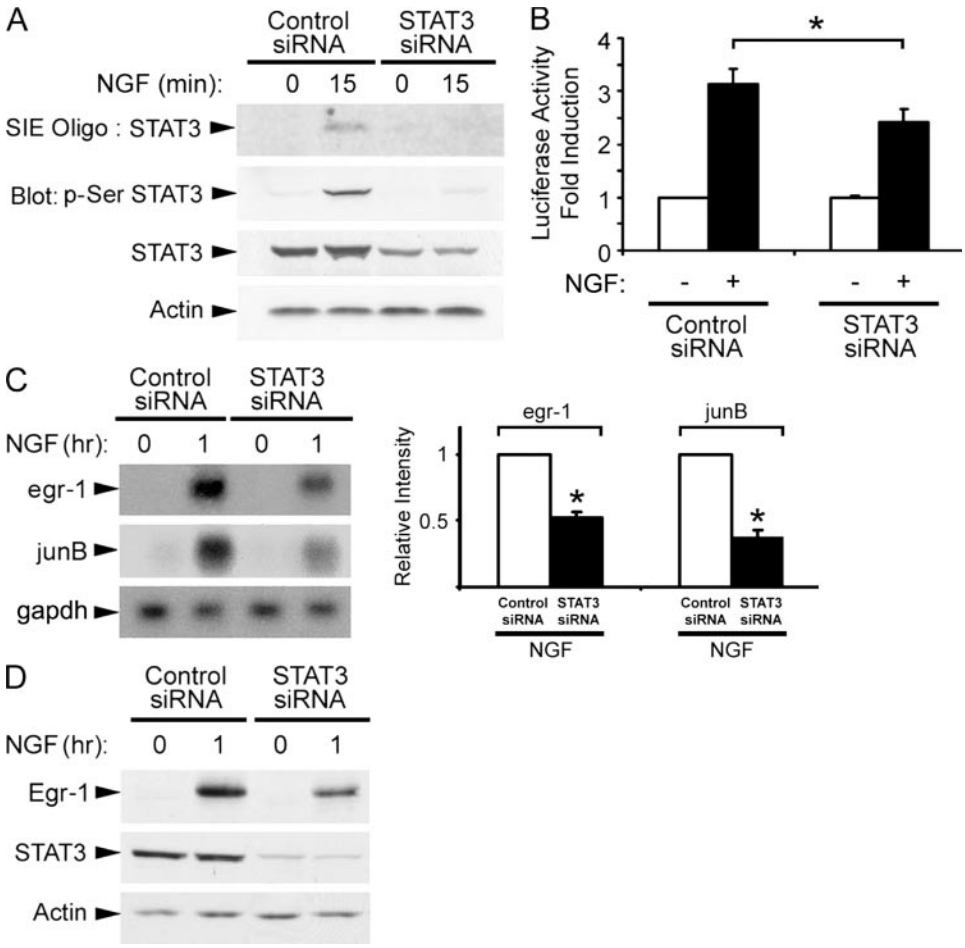

FIGURE 4. **NGF-mediated STAT3 DNA binding and transcriptional activity was markedly attenuated by STAT3 siRNA.** *A*, PC12 cells were transfected with control RNA oligo or STAT3 siRNA. After overnight incubation, STAT3 DNA binding activity in the transfected cell lysates were tested using the SIE-agarose beads. *B*, PC12 cells were transfected with control siRNA or STAT3 siRNA the day before transfection with pSTAT3-Luc reporter and then subjected to the promoter luciferase assay as in Fig. 1; mean ± S.E., $n = 3$; *, $p < 0.05$. *C*, the mRNA expression of NGF-induced genes, *egr-1* and *junB*, after STAT3 siRNA transfection was analyzed by Northern blotting. Quantitation shown represents the relative intensity to the control siRNA-transfected cells treated with NGF from three separate experiments. *D*, NGF-induced Egr-1 protein expression in STAT3 siRNA-transfected PC12 cells.

early genes including *egr-1* and *junB*, which are crucial for the functions of NGF in PC12 cells.

Reduction of STAT3 Expression Attenuated NGF-induced Cyclin D1 Expression and Promoted PC12 Cell Proliferation in the Presence of NGF—To further elucidate the functional roles of STAT3 in NGF-induced differentiation in PC12 cells, we searched for genes implicated in PC12 cell differentiation that are potentially regulated by both NGF and STAT3. Interestingly, we found that expression of *cyclin D1* mRNA and protein was attenuated in STAT3 siRNA-transfected PC12 cells upon NGF treatment (Figs. 5, *A* and *B*). The results obtained therefore suggest that STAT3 is not only required for expression of immediate early genes during initiation of differentiation, but is also required for expression of late response genes during the differentiation process.

NGF treatment in PC12 cells initially promotes a transient phase of proliferation, although it subsequently leads to G_0/G_1 growth arrest and induces neuronal differentiation. During differentiation of PC12 cells, cell cycle withdrawal is accompanied by the induction of a G_1-phase cyclin, cyclin D1 (40, 45, 46). Ectopic expression of cyclin D1 in PC12 cells induces cell cycle withdrawal (46). The attenuated expression of cyclin D1 in STAT3 siRNA-transfected PC12 cells after NGF treatment prompted us to investigate if the NGF-induced cessation of cell proliferation was also inhibited after reduction of STAT3 level. Cell proliferation of the transfected PC12 cells was determined by counting the number of viable cells and 5-bromo-2′-deoxyuridine labeling after NGF treatment for up to 4 days. Remarkably, STAT3 siRNA transfection resulted in a significant increase in total cell number in the presence of NGF as compared with the control (Fig. 5*C*). Moreover, incorporation of 5-bromo-2′-deoxyuridine was markedly enhanced in STAT3 siRNA-transfected PC12 cells after 4 days of NGF treatment, indicating that NGF-triggered cessation of cell proliferation was attenuated when STAT3 level was reduced. Together, these observations suggest that NGF-induced cell cycle withdrawal in PC12 cells acted at least in part through the activation of STAT3 by inducing the expression of cyclin D1.

Attenuation of STAT3 Expression Abolished BDNF-promoted Neurite Extension in Cultured Hippocampal Neurons—Given the observation that STAT3 could affect PC12 cell proliferation before neurite outgrowth during neuronal differentiation, the specific effect STAT3 might exhibit toward neurite outgrowth in PC12 cells could be masked by its effect on proliferation. Thus, to examine if neurotrophin-induced STAT3 activation may specifically affect neurite extension, the effect of STAT3 on BDNF-induced neurite outgrowth in primary hippocampal neurons was investigated. BDNF has been observed to increase neurite outgrowth and arborization in cultured E18 hippocampal neurons and in the hippocampus of BDNF-overexpressing transgenic mice (47, 48). E18 hippocampal neurons at DIV2 were transfected with control or STAT3 siRNA together with green fluorescent protein-expressing vector and then maintained in culture medium with or without BDNF supplement for 2 days. These cultures were fixed, and the length of the longest neurite was measured in each condition. In accordance with previous observation (47), BDNF induced a significant increase in the length of the longest neurite in cultures transfected with control siRNA. Interestingly, the length of the longest neurite was comparable in cultures transfected with STAT3 siRNA in the presence or absence of BDNF (Figs. 6, *A* and *B*), indicating that attenuation of STAT3 level (Fig. 6*C*) almost

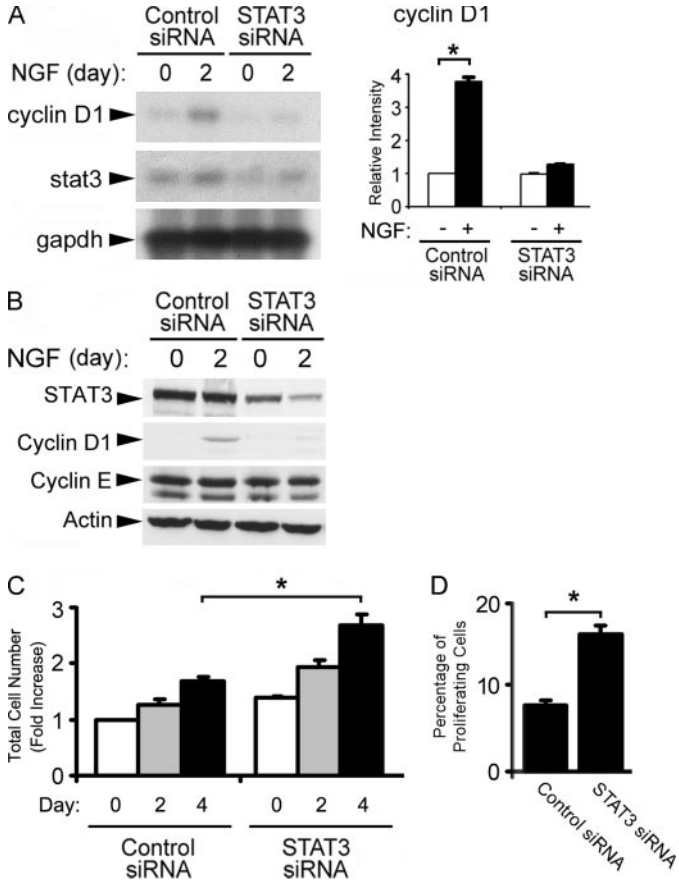

FIGURE 5. **NGF-induced cyclin D1 expression and reduction of PC12 cell proliferation was attenuated by STAT3 siRNA.** *A*, PC12 cells were transfected with control RNA oligo or STAT3 siRNA. After overnight incubation, cells were treated with NGF for 2 days. The mRNA expression of *cyclin D1* was analyzed by reverse transcription-PCR analysis. *B*, expression of cyclin D1 and cyclin E protein in STAT3 siRNA-transfected PC12 cells treated with NGF. *C*, total cell count in the control and STAT3 siRNA transfected PC12 cells after treatment with NGF for 2 to 4 days. *D*, quantitation of cell proliferation (5-bromo-2′-deoxyuridine staining) in the control and STAT3 siRNA-transfected PC12 cells after 4 days of NGF treatment; mean ± S.E., $n = 3$; *, $p < 0.05$.

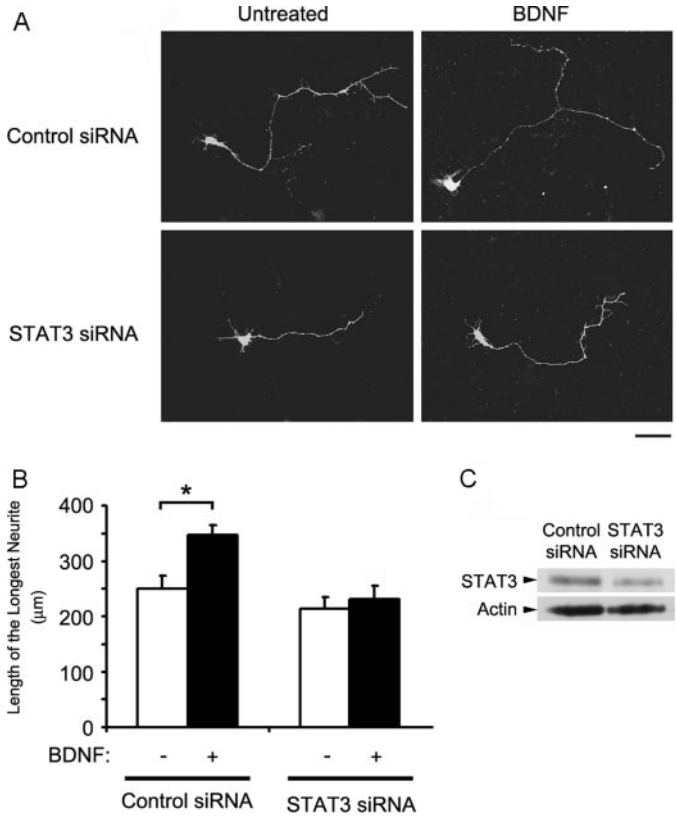

FIGURE 6. **Reduction of STAT3 expression attenuated BDNF-induced neurite outgrowth of cultured hippocampal neurons.** *A*, cultured hippocampal neurons at DIV2 were transfected with the control and STAT3 siRNA together with green fluorescent protein expressing vectors. Twenty-four hour after transfection, cells were left untreated or treated with BDNF for 2 days. *Scale bar*, 50 μm. *B*, quantitation of the length of the longest neurite; mean ± S.E., $n = 3$; *, $p < 0.05$. *C*, STAT3 expression in STAT3 siRNA-transfected primary hippocampal neurons was analyzed by Western blotting.

abrogated the induction of neurite extension by BDNF. Apart from the length of the longest neurite, we did not observe other morphological differences in cells transfected with STAT3 siRNA. Thus, activation of STAT3 was apparently required for BDNF-induced neurite extension in cultured hippocampal neurons.

DISCUSSION

Originally identified as a transcription factor downstream of cytokine stimulation and Janus kinase activation, STAT3 has become an increasingly important signal transducer due to the implication of STAT3 activation in a myriad of signaling cascades. For example, phosphorylation and activation of STAT3 has been observed to occur via various RTKs, such as the receptors of epidermal growth factor, platelet-derived growth factor, neuregulin, and ephrins (11–14, 49, 50). Here we add to the list of RTKs that triggers STAT3 activation by identifying STAT3 as a downstream signaling component of Trk activation. We report that treatment of PC12 cells with NGF resulted in TrkA-dependent STAT3 activation, which in turn contributed to NGF-induced gene transcription. Furthermore, we observed that treatment of cortical neurons with BDNF similarly induced STAT3 activation through stimulating serine and tyrosine phosphorylation of STAT3. More importantly, activation of STAT3 by NGF regulated the expression of cyclin D1, which was required for NGF-stimulated cell cycle withdrawal. We also provided evidence that reduction of STAT3 expression in cultured hippocampal neurons attenuated BDNF-mediated neurite extension. These observations collectively reveal that STAT3 may serve as a signal transducer for Trk receptors in neuronal cells and that neurotrophin-induced activation of STAT3 may play crucial roles in the downstream functions of neurotrophin signaling.

Despite the increasing implications of STAT3 in RTK signaling, the mechanisms through which STAT3 is recruited after RTK activation are far from clear. Ephrin A4 signaling has been suggested to phosphorylate STAT3 through Janus kinase 2 activation (13). However, both epidermal growth factor receptor and platelet-derived growth factor receptor can phosphorylate STAT3 via a Janus kinase-independent pathway (49, 50). Neuregulin/ErbB-induced Tyr-705 phosphorylation of STAT3, on the other hand, is mediated by Src (11). Although the mechanisms by which STAT3 is activated after neurotrophin stimulation remained unknown, we demonstrated that NGF-induced STAT3 activation required TrkA activation. In addition, we showed that Ser-727 phosphorylation of STAT3 in PC12 cells occurred through both H7-sensitive and H7-insensitive pathways. Pathways implicated in interleukin-6-activated STAT3 serine phosphorylation can be distinguished into H7-sensitive and H7-insensitive pathways through different motifs in the gp130 receptor (26). H7-sensitive kinases include protein kinase C (PKC), cAMP- and cGMP-dependent protein kinase (PKA and PKG) and myosin light chain kinase (MLCK), but do not involve Erk, p38, JNK, or protein kinase Cδ. Although NGF-induced STAT3 Ser-727 phosphorylation was sensitive to H7 and involved Erk in naïve PC12 cells,

it was insensitive to H7 in primed cells. Rather, Erk, phosphatidylinositol 3-kinase, and active Cdk5 were implicated in 1 day-NGF-primed PC12 cells. Thus, phosphorylation of STAT3 at serine 727 might function as a convergent point for several signaling pathways triggered by Trk activation. Further studies will undoubtedly provide important insights on how these signaling pathways co-operate to regulate STAT3 phosphorylation, in particular in response to RTK stimulation.

In the current study we observed that although NGF induced Ser-727 phosphorylation of STAT3, phosphorylation of STAT3 at Tyr-705 was hardly detectable during the first 30 min of NGF treatment in PC12 cells. Although Tyr-705 phosphorylation of STAT3 is generally regarded as the prerequisite for STAT3 activation, our observations demonstrated the importance of Ser-727 phosphorylation in STAT3 function in the absence of detectable Tyr-705 phosphorylation in PC12 cells. Both STAT3 DNA binding and transcriptional activities were induced in PC12 cells upon NGF treatment where only Ser-727-phosphorylated STAT3 was observed. More importantly, overexpression of STAT3 mutant lacking Ser-727 almost completely abolished the NGF-induced STAT3 transcriptional activity. In corroboration with our findings, Ser-727 phosphorylation of STAT3, but not Tyr-705, mediates the expression of survival signal Mcl-1 (a member in the Bcl-2 family) in macrophages (52). Furthermore, Ser-727 phosphorylation of STAT3 is observed after stimulation by insulin, granulocyte-macrophage colony-stimulating factor, and interleukin-3 in the absence of Tyr-705 phosphorylation (18–20). The importance of Ser-727 phosphorylation of STAT3 is further highlighted in the reduced body size and reduced survival of thymocytes in STAT3 mutant mice harboring only one allele of STAT3, where Ser-727 was mutated to alanine (51). Taken together, our findings lend additional support to the notion that Ser-727-phosphorylated STAT3 plays a crucial role in the activation of STAT3. The lack of observable Tyr-705 phosphorylated STAT3 in NGF-treated PC12 cells reported here could be due to the presence of a potential intrinsic dephosphorylation mechanism that negatively regulates the NGF-induced Tyr-705 phosphorylation. Alternatively, phosphorylation of Ser-727 may inhibit Tyr-705 phosphorylation in naïve PC12 cells, similar to that observed in other cellular systems (19, 21, 22). Furthermore, Ser-727 of STAT3 is crucial for its recruitment of p300 upon cytokine stimulation (17, 37–39). Indeed, we found that NGF-induced phosphorylation of STAT3 at Ser-727 was accompanied by concomitant binding of STAT3 to p300. Our observations thus corroborate with other findings by showing that NGF stimulation also induced STAT3 and p300 association, thereby activating gene transcription.

In the current study we observed that neurotrophin-induced activation of STAT3 is required for several downstream functions of neurotrophin signaling. First of all, NGF-induced STAT3 phosphorylation mediated NGF-dependent gene transcription. The levels of NGF-specific differentiation transcripts, for example, *egr-1*, *junB*, and *cyclin D1*, were decreased when STAT3 function was attenuated by RNA interference. Indeed, reduction of the STAT3 level inhibited NGF-induced growth arrest in PC12 cells. Furthermore, attenuation of STAT3 levels abrogated BDNF-promoted neurite extension in cultured hippocampal neurons. Although the precise mechanisms involved remain to be delineated, our results revealed a novel biological role of STAT3 as an important component in neurotrophin signaling and functions in both PC12 cells and differentiated neurons. Investigations on the potential involvement of STAT3 activation in other functions of growth factor/RTK signaling will shed light on the functional significance of this increasingly important signal transducer in RTK signaling.

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