Ciliary Neurotrophic Factor Stimulates the Phosphorylation of Two Forms of STAT3 in Chick Ciliary Ganglion Neurons*

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Ciliary neurotrophic factor (CNTF) is a neuropoietic cytokine that was identified, purified, and cloned based on its neurotrophic activity on cultured chick ciliary ganglion neurons. The molecular mechanisms by which CNTF elicits its effects on these neurons are unknown. We have previously identified functional receptors for CNTF on ciliary ganglion neurons and demonstrated the CNTF-specific tyrosine phosphorylation of an approximately 90-kDa protein. Here we show that CNTF induced the rapid tyrosine phosphorylation and nuclear accumulation of this protein and identify it as an avian form of the transcription factor, STAT3. Identification was confirmed by its recognition with two distinct anti-STAT3 antibodies and the lack of binding to antibodies against STAT1, -2, -4, -5, or -6. The phosphorylation was stable for up to 2 h but required the continued presence of CNTF. CNTF also induced the tyrosine phosphorylation of a similar protein in cultured chick dorsal root ganglion and retinal neurons. In addition, we identify a second, 100-kDa form of STAT3 that appears in response to CNTF. Unlike previous reports, utilizing mammalian cell lines that detected a slower migrating form of STAT3 resulting from H7-sensitive protein phosphorylation, H7 did not prevent the appearance of the 100-kDa form in ciliary neurons. Thus, the 100-kDa avian protein may represent a novel form of CNTF-inducible STAT3.

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1 The abbreviations used are: CNTF, ciliary neurotrophic factor; ECL, enhanced chemiluminescence; GPA, growth-promoting activity; MEM, Eagle’s minimal essential medium.
phorylation of this 90-kDa protein, therefore, represents a potential signal transduction pathway activated specifically by mammalian CNTF and chick GPA. In this study we show for the first time in primary chicken neurons that the 90-kDa phosphoprotein is translocated to the nucleus and is immunologically identical with the transcription factor, STAT3. In addition, CNTF induces a modified 100-kDa form of STAT3 that appears distinct from previously described mammalian serine-phosphorylated STAT3 (14).

**EXPERIMENTAL PROCEDURES**

**Cell Culture**—Ciliary ganglion neurons were dissociated from 8-day-old chick embryos and grown at 37 °C in a 5% CO₂, 95% air atmosphere as described previously (20). Cells for the tyrosine phosphorylation assays were plated on 35-mm culture dishes on a substratum of poly-d-lysine and mouse laminin. The culture medium consisted of Eagle's minimal essential medium (MEM) containing 10% (v/v) heat-inactivated horse serum, 50 units/ml penicillin, and 50 mg/ml streptomycin. The medium was adjusted to a final concentration of 25 mM KCl, which activated horse serum, 50 units/ml penicillin, and 50 mg/ml streptomycin. Freshly dissociated retina from 11-day-old embryos were trypsin-digested (0.25%) for 30 min at 37 °C and then processed as described for ciliary ganglion neurons except that cells were plated on tissue culture dishes coated with poly-d-ornithine (10 μg/cm²) at a final density of 5 × 10⁵ cells/cm² in MEM with 10% horse serum. Dorsal root ganglion neurons from 11-day-old embryos were cultured on a substratum of rat tail collagen in medium supplemented with 100 ng/ml nerve growth factor, as described previously (30).

Cell survival was determined on ciliary ganglion neurons by using a 1-(4,4-dimethyl thiazol-2-yl)-2,5-diphenyl tetrazolium bromide dye assay as described previously (20, 31).

**Immunoblot Detection of Proteins**—Ciliary ganglion neurons were grown in culture for 5 days, rinsed, and incubated in serum-free, unsupplemented medium for 3 h; the neurons were then treated for the indicated periods with human CNTF (2 nM, 50 ng/ml unless otherwise indicated, dissolved in phospho-free MEM containing 1% Na₂VO₄, and stored at −80 °C). The proteins were separated on a 5% SDS-polyacrylamide gel and then transferred to nitrocellulose. The membranes were blocked for 2 h in Tris-buffered saline (10 mM Tris-HCl, pH 7.5, 137 mM NaCl, and 0.05% Tween 20) with 1% ovalbumin (or 5% bovine serum albumin) and incubated for 4 h with anti-phospho-tyrosine monoclonal antibody 4G10 (1:1000) as described previously (20, 21). Where indicated, cultures were treated for 20 min with 0.1 or 1 μM staurosporine, for 30 min with 200 μM H7, or for 5 min with 20 mM sodium fluoride prior to the treatment with CNTF. The proteins were separated on electrophoresis on a 5% SDS-polyacrylamide gel and then transferred to nitrocellulose. The membranes were blocked for 2 h in Tris-buffered saline (10 mM Tris-HCl, pH 7.5, 137 mM NaCl, and 0.05% Tween 20) with 1% ovalbumin (or 5% bovine serum albumin) and incubated for 4 h with anti-phospho-tyrosine monoclonal antibody 4G10 (1:1000) as described previously (20, 21). With a monoclonal antibody to STAT3 (1:1000) (13), a polyclonal antibody directed against tyrosine-phosphorylated Tyr-705 STAT3 (1:1000) (13), or other anti-STAT monoclonal antibodies as recommended by the supplier. Following incubation with either goat anti-mouse IgG or anti-rabbit IgG conjugated to horseradish peroxidase the reactive proteins were visualized using enhanced chemiluminescence (ECL) according to the manufacturer's instruction (Amersham Life Sciences, Inc.). The level of antibody binding was quantified by scanning densitometry (Ultrascan XL, Pharmacia Biotech Inc.). In some experiments the nitrocellulose membranes were stripped and analyzed using a different primary antibody as described in the ECL Manual (Amerlsham Life Sciences, Inc.).

**Immunoprecipitation**—Ciliary ganglion neurons (3.4 × 10⁵ cells; 50 ganglion eq) grown for 5 days in culture were serum-starved for 3 h at 37 °C in MEM and then treated for 5 min with 2 nM CNTF. Cells were rinsed, solubilized with 50 μl of boiling 1% SDS in 10 mM Tris-HCl, pH 7.4, and then diluted with 150 μl of 2× cold immunoprecipitation buffer (1× buffer is 150 mM NaCl, 1% Triton X-100, 0.5% Nonidet P-40, 1 mM EDTA, 1 mM EGTA, 1 mM Na₃VO₄, 0.5 mM phenylmethylsulfonyl fluoride, 20 μg/ml leupeptin, 20 μg/ml aprotinin, 10 mM Tris base, pH 7.4) and 120 μl of H₂O. Anti-STAT3 monoclonal antibody or nonimmune mouse serum was added to cell lysates (1:100) and incubated for 1 h at 4 °C. Rabbit anti-mouse IgG (Cappel) was then added for an additional 30 min and the antibody-bound proteins were captured with protein A, washed twice with immunoprecipitation buffer and washed twice with immunoprecipitation buffer and then solubilized in boiling Laemmli sample buffer containing 2 mM Na₃VO₄. The soluble proteins after the immunoprecipitation with anti-STAT3 antibodies were precipitated with 6 volumes of ice-cold acetone, and the dried pellet was solubilized in boiling Laemmli sample buffer containing 2 mM Na₃VO₄.

**Cell Fractionation**—Crude nuclear and cytoplasmic extracts were prepared as described (32). Ciliary ganglion neuron cultures were washed and lysed by homogenization in hypotonic buffer (20 mM HEPES, pH 7.0, 10 mM KCl, 1 mM MgCl₂, 0.1% Nonidet P-40, 10% glycerol, 0.5 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride, 1 mM Na₃VO₄, 2 μg/ml leupeptin). Nuclei were separated by centrifugation at 200 × g, and the pelleting the nuclei was washed twice in hypotonic buffer without Nonidet P-40 before immunoblot analysis as described above. The cytoplasmic fraction (the supernatant from the first centrifugation) was clarified by centrifugation at 14,000 × g, and the proteins were precipitated with acetic acid and subjected to immunoblot analysis as above.

**Materials**—Embryonated chick eggs (mixed heavy breed, Rhode Island Red/Barred Rock/White Rock) were obtained from Gawlak Farms (Lawton, NY) and grown at 37 °C in a humidified incubator. Cell culture reagents and molecular weight markers were obtained from Life Technologies, Inc. Laminin was from Collaborative Biomedical Products (Bedford, MA), staurosporine and H7 were from Calbiochem, and H7 was also obtained from Seikagaku America (Rockville, MD). Monoclonal antibody 4G10 was from Upstate Biotechnology (Lake Placid, NY), polyclonal anti-phospho-STAT3 antibody was from New England Biolabs (Beverly, MA), and monoclonal antibodies to all other STATs were from Transduction Laboratories (Lexington, KY). Recombinant human CNTF was generously provided by Regeneron Pharmaceuticals (Tarrytown, NY).

**RESULTS**

**Nuclear Translocation of a 92-kDa CNTF-induced Tyrosine-phosphorylated Protein**—CNTF induced the phosphorylation of an approximately 90-kDa protein in ciliary ganglion neurons that corresponded to the size described for members of the STAT family of transcription factors (Mₚ = 84,000–113,000) (10, 20). Thus we determined whether this CNTF-induced tyrosine-phosphorylated protein from ciliary ganglion neurons was translocated from the cytoplasm to the nucleus as required for transcriptional activity. A tyrosine-phosphorylated 92-kDa protein was detected at very low levels in the nucleus before CNTF stimulation; however, after 15 min of CNTF treatment the 92-kDa phosphoprotein had accumulated in the nuclear fraction (Fig. 1, left panel). Sodium fluoride (20 mM), an inhibitor of nuclear translocation of activated STAT1 (33), inhibited 80% of the CNTF-induced nuclear translocation of the 92-kDa phosphoprotein (Fig. 1, left panel). These results indicated that the CNTF-induced tyrosine-phosphorylated protein was translocated to the nucleus by a fluoride-sensitive mechanism.

**Recognition of the 92-kDa Protein by Anti-STAT3 Antibodies**—The STAT family of transcription factors includes six known members through which cytokines can differentially modulate gene transcription. The CNTF family of cytokines has been reported to activate both STAT1 and STAT3 (11). We examined cell lysates of cultured ciliary ganglion neurons using antibodies against STAT1, -2, -3, -4, -5, or -6 to determine if similar sized products were detectable. Only antibodies to STAT3 produced a visible reaction at 92 kDa (1 ± kDa, n = 6) in ciliary ganglion neurons (Fig. 2A). CNTF treated ciliary ganglion neurons, a doublet of about 112/119 and 105 kDa, were detected using anti-STAT3 and -6 antibodies, respectively. In control experiments we used a test for cross-reactivity of STAT1, -2, and -4 with chick proteins, antibodies to STAT1 detected a doublet of 93/95 kDa in lysates from chick embryo fibroblasts, and the STAT2 antibody detected a band of 90 kDa in chick embryo spleen (data not shown). Therefore, these antibodies, except for anti-STAT4, recognize chick proteins comparable in size with those seen in mammalian cells. To confirm the identity of the 92-kDa product as STAT3 as compared with STAT1 we used antibodies specific for STAT1 and STAT3 to precipitate proteins from extracts of CNTF-treated ciliary ganglion neurons. Monoclonal antibodies to STAT3 immunoprecipitated proteins of 92 kDa (Fig. 2B). In contrast, anti-STAT1 antibodies and nonimmune mouse serum failed to immunoprecipitate the 92-kDa protein (data not shown).
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Fig. 1. CNTF-induced nuclear translocation of a 92-kDa tyrosine-phosphorylated protein. Ciliary ganglion neurons from 8-day-old chick embryos were plated and grown for 5 days in 25 mM KCl medium. Nuclear fractions were prepared from untreated cultured neurons (lane 1) or following CNTF treatment for 15 min (lanes 2 and 3) as described under “Experimental Procedures.” NaF treatment (20 mM) was performed for 5 min (lane 3) prior to CNTF stimulation. Immunoblots of proteins from nuclear fractions were probed with monoclonal antibody PY4G10 (left panel), visualized by ECL, and then stripped and reprobed with anti-STAT3 antibody (right panel) and visualized again by ECL. NaF treatment resulted in a 73 ± 7% (n = 2, ± range) inhibition of the nuclear translocation of the 92-kDa phosphoprotein detected by PY4G10 as assessed by densitometry scanning and normalization to the constitutively phosphorylated proteins migrating at 103 and 130 kDa (indicated by asterisks). The position of the 92-kDa protein is indicated by arrows, and the positions of molecular size standards are shown as bars in the center (215,000, 105,000, 70,000, and 43,000).

Fig. 2. The CNTF-induced 92-kDa phosphotyrosylated protein cross-reacted with anti-STAT3 antibodies. A, cell lysates from ciliary ganglion neurons were separated by SDS-gel electrophoresis and immunoblotted using antibodies to STAT1–6 (lanes 1–6, respectively). Shown are the 60–120-kDa (bars are positions of 105,000 and 70,000 standards) regions of the blots following ECL. B, ciliary ganglion neurons were either untreated (control, lanes 1, 3, 5, and 7) or treated for 5 min with 2 nM CNTF (lanes 2, 4, 6, and 8). Proteins from whole cell lysates were immunoprecipitated with monoclonal anti-STAT3 (1:100, lanes 1–4), and the immunoprecipitated proteins were subjected to immunoblot analysis with the anti-STAT3 antibody (lanes 1 and 2). The membrane was then stripped and reprobed with the anti-phosphotyrosine antibody 4G10 (lanes 3 and 4). Proteins remaining in the supernatant following immunoprecipitation were acetone-precipitated, immunoblotted, and probed with the anti-STAT3 antibody (lanes 5 and 6) and then stripped and reprobed with antibody 4G10 (lanes 7 and 8). Relative mobility of the 92-kDa protein is indicated by arrows. In a replicate experiment (C), lots of anti-STAT3 immunoprecipitated material (lanes 1, 2, 4, and 5) as described in panel B or normal mouse serum immunoprecipitated material (lanes 3 and 6) were first probed with anti-STAT3 antibodies (lanes 1–3) and then stripped and reprobed with an anti-phospho-STAT3 antibody (lanes 4–6) and exposed after each to film for an extended time (90 and 20 min, respectively) to reveal both 92- and 100-kDa (arrow) forms of STAT3.

Characterization of CNTF-induced Tyrosine Phosphorylation of STAT3—Protein tyrosine phosphorylation initiated by ligand-receptor interaction is usually described as a rapid and transient event typically occurring within 1–5 min and declining after 30–60 min (14). Time course experiments of CNTF-induced tyrosine phosphorylation of the 92-kDa protein in cultured ciliary ganglion neurons showed unusual stability. Increased tyrosine phosphorylation of the 92-kDa protein was detectable within 3 min of CNTF exposure and increased up to 15 min and remained at elevated levels during 2 h of continuous exposure (Fig. 3A). To further confirm the identity of the 92-kDa phosphoprotein we used a recently developed antibody from New England Biolabs, Inc. that specifically recognizes tyrosine-phosphorylated (Tyr-705) STAT3. The time course of tyrosine phosphorylation was similar whether determined using the anti-phosphotyrosine (4G10) or the anti-phospho-

STAT3 antibodies, and little change in total STAT3 protein was observed with continued CNTF treatment (Fig. 3A). These results provide strong evidence that the 92-kDa protein represents avian STAT3. A lighter intensity band of 100 kDa (± 1 kDa, n = 4) was visualized in material prepared from CNTF-treated cells when immunoblots probed with the anti-STAT3 antibody were re-exposed to film for longer periods (Fig. 3A, inset).

CNTF has effects on a variety of chick neuronal types in both the peripheral and central nervous systems. The CNTF-mediated activation of STAT3 was not limited to parasympathetic neurons of ciliary ganglia as cells cultured from both dorsal root ganglia and neural retina showed a 2-fold increase in tyrosine phosphorylation of STAT3 after 10 min of CNTF treatment (Fig. 4). Thus, other neurons whose in vitro survival is supported by CNTF, dorsal root ganglion neurons (35), as well as neurons whose cholinergic development is regulated in vitro by CNTF, neural retina (36), also show tyrosine phosphorylation of STAT3.

The survival of cultured ciliary ganglion neurons requires...
the continued presence of trophic factor support (37). The tyrosine phosphorylation of STAT3 also required the continued presence of CNTF. Ciliary ganglion neurons were treated with CNTF for 5 min; the neurons were then rinsed and incubated with CNTF-free medium. After 1 h of CNTF deprivation the level of tyrosine phosphorylation returned to near prestimulation levels (Fig. 3B). These results indicated that maintenance of the tyrosine phosphorylation of STAT3 depended on the continued presence of CNTF. In addition, chronic growth in CNTF did not eliminate the response. Neurons grown in either 25 mM KCl medium, 1 mM CNTF, or 10 mM basic fibroblast growth factor/heparin for 4 days, each of which promotes survival of ciliary neurons, followed by incubation of cells for 3 h in unsupplemented medium and stimulation with 5 mM CNTF induced tyrosine phosphorylation of STAT3 (data not shown).

**DISCUSSION**

**Identification of STAT3 in Ciliary Ganglion Neurons**—We have previously shown that CNTF stimulation of ciliary ganglion neurons resulted in the rapid induction of a 92-kDa tyrosine-phosphorylated protein (20). The 92-kDa phosphoprotein was the only major phosphoprotein that consistently showed a specific increase in tyrosine phosphorylation in re-
Immunoblots of cell proteins were analyzed with anti-phospho-STAT3 and anti-STAT3 antibodies (bottom). Similar results were obtained in two additional experiments. Cells were untreated or treated with 1 μM to 10 nM CNTF as indicated. Immunoblots of cellular proteins were probed with anti-phospho-STAT3 antibodies (top) and then stripped and reprobed with anti-STAT3 antibodies (bottom) and visualized by ECL. The blots were exposed normally during 10 min with 2 nM CNTF (lanes 2–4) or untreated (lane 1). Immunoblots of cell proteins were analyzed with anti-phospho-STAT3 antibodies (top), visualized by ECL, and then stripped and reprobed with anti-STAT3 antibodies (bottom) and visualized by ECL again. Similar results were obtained in two additional experiments. Cells were untreated (lanes 1 and 4) or treated with CNTF (lanes 2 and 3) after incubation with 200 μM H7 for 30 min (lanes 3 and 4). Immunoblots of cell proteins were analyzed with anti-phospho-STAT3 antibodies and visualized by ECL. The blots were exposed normally during ECL to detect the 92-kDa STAT3 band (arrow, 15 s) or overexposed to detect the 100-kDa STAT3-immunoreactive product (arrowhead, 5 min).

**FIG. 5.** CNTF stimulated the appearance of an additional 100-kDa STAT3-like product. A, cells were incubated with staurosporine (Stauro, 1.0 and 0.1 μM) for 20 min (lanes 3 and 4) and subsequently treated for 10 min with 2 nM CNTF (lanes 2–4) or untreated (lane 1). Immunoblots of cell proteins were analyzed with anti-phospho-STAT3 antibodies (top), visualized by ECL, and then stripped and reprobed with anti-STAT3 antibodies (bottom) and visualized by ECL again. Similar results were obtained in two additional experiments. B, cells were untreated (lanes 1 and 4) or treated with CNTF (lanes 2 and 3) after incubation with 200 μM H7 for 30 min (lanes 3 and 4). Immunoblots of cell proteins were analyzed with anti-phospho-STAT3 antibodies and visualized by ECL. The blots were exposed normally during ECL to detect the 92-kDa STAT3 band (arrow, 15 s) or overexposed to detect the 100-kDa STAT3-immunoreactive product (arrowhead, 5 min).

**FIG. 6.** Concentration dependence of CNTF induction of tyrosine-phosphorylated 92- and 100-kDa forms of STAT3. Cells were untreated or treated with 1 μM to 10 nM CNTF as indicated. Immunoblots of cellular proteins were probed with anti-phospho-STAT3 antibodies (top) and then stripped and reprobed with anti-STAT3 antibodies (bottom) and visualized by ECL. The blots were exposed normally (20 and 5 s, respectively) to detect the 92-kDa STAT3 band (arrow) or overexposed (5 and 2 min, respectively) to detect the 100-kDa STAT3-immunoreactive product (arrowhead). Equivalent results were obtained in a replicate experiment.

**Characterization of STAT3 Phosphorylation—**Mammalian STAT3 shares 40–50% amino acid identity to STAT1 and STAT2 and recognizes a DNA binding element that resembles the consensus sequence found for STAT1, -4, -5, and -6 and responds to physiologically relevant concentrations of human CNTF and avian GPA but was not induced by a series of CNTF-related cytokines (19) (interleukin-6, interleukin-11, and oncostatin M). In this study we identified the 92-kDa tyrosine-phosphorylated protein in ciliary ganglion neurons as a member of the STAT family of transcription factors based on three criteria. First, the 92-kDa protein underwent tyrosine phosphorylation and translocation from the cytoplasm to the nucleus following CNTF stimulation, a process necessary for DNA binding and gene regulation by STATs. Second, the nuclear translocation of the 92-kDa phosphoprotein was inhibited by sodium fluoride, which also inhibits activated STAT nuclear translocation in response to interferons and epidermal growth factor (32, 33). Finally, the 92-kDa tyrosine-phosphorylated protein was immunoprecipitated with a monoclonal antibody specific for the carboxyl terminus of mammalian STAT3 and was recognized by a polyclonal antibody specific for tyrosine-phosphorylated (Tyr-705) STAT3. A second 100-kDa tyrosine-phosphorylated protein was induced by CNTF and recognized by both STAT3 antibodies. There was no evidence for cross-reactivity of either the 92- or 100-kDa proteins with antibodies to STAT1, -2, -4, -5, or -6. Although these antibodies were reactive with either the 92-kDa STAT3 protein in cell lysates recognized by both the STAT3 and phospho-STAT3 antibodies. This CNTF-induced shift of apparent molecular mass of 8 kDa was significantly greater than the 2-kDa shift observed in mammalian cell lines (14, 15). In cell lines, cytokines induce a time-dependent shift of STAT3f to STAT3s, whereas in ciliary ganglion neurons the 100-kDa form remained a small percentage of the total STAT3 immunoreactive pool even at higher CNTF concentrations and after extended treatment. It is yet to be determined if the newly generated 100-kDa pool is static or is actively turning over. A final difference between STAT3s and the chick 100-kDa STAT3 is the lack of sensitivity to H7 by the latter. Two different sources of H7 were unable to prevent the appearance of the 100-kDa STAT3, whereas H7 effectively prevented the conversion of STAT3f to STAT3s in CNTF-treated SH-SY5Y human neuroblastoma cells. Additionally, H7 blocked CNTF and high KCl-mediated cell survival in cultured ciliary ganglion neurons in control experiments, indicating that H7 was active in these

**Drosophila STAT** (10). However, while STAT1 is activated by a number of cytokines and growth factors, STAT3 is activated mainly by the CNTF family of cytokines and epidermal growth factor and is implicated in the regulation of transcription of acute phase protein genes in liver cells. The gene for vasoactive intestinal peptide in rat also contains a cytokine response element (10, 12, 38, 39). The specificity of STAT3 activation, at least in part, is achieved by the tyrosine-based motifs in gp130 and LIFRβ, the signal transducer receptor components for the CNTF family of cytokines (40). Recently, two cytokine-induced tyrosine-phosphorylated forms of STAT3 have been characterized, STAT3f and STAT3s, differing in H7-sensitive secondary serine/threonine phosphorylation (14). The two forms differ in apparent molecular mass by 2 kDa, undergo nuclear translocation, and recognize the same DNA sequences in vitro but may act on distinct target genes in vivo, depending on the cell type (14). In ciliary ganglia we found that CNTF induced the appearance of an additional 100-kDa form of tyrosine-phosphorylated STAT3. We do not know if it was generated by a secondary modification of the 92-kDa STAT3. However, the 92-kDa STAT is the most likely source since it is the only other protein in cell lysates recognized by both the STAT3 and phospho-STAT3 antibodies. This CNTF-induced shift of apparent molecular mass of 8 kDa was significantly greater than the 2-kDa shift observed in mammalian cell lines (14, 15). In cell lines, cytokines induce a time-dependent shift of STAT3f to STAT3s, whereas in ciliary ganglion neurons the 100-kDa form remained a small percentage of the total STAT3 immunoreactive pool even at higher CNTF concentrations and after extended treatment. It is yet to be determined if the newly generated 100-kDa pool is static or is actively turning over. A final difference between STAT3s and the chick 100-kDa STAT3 is the lack of sensitivity to H7 by the latter. Two different sources of H7 were unable to prevent the appearance of the 100-kDa STAT3, whereas H7 effectively prevented the conversion of STAT3f to STAT3s in CNTF-treated SH-SY5Y human neuroblastoma cells. Additionally, H7 blocked CNTF and high KCl-mediated cell survival in cultured ciliary ganglion neurons in control experiments, indicating that H7 was active in these

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cells. Therefore, the 100-kDa band appears to be a novel STAT3 induced in chick neurons with distinct properties from those of the serine-phosphorylated STAT3 previously identified in cell lines. The question remains whether it represents an active form or a form associated with signal termination.

The CNTF-induced tyrosine phosphorylation of STAT3 in ciliary ganglion neurons was unusually stable. Analysis of the time course of the tyrosine phosphorylation showed that the effect was induced rapidly after CNTF stimulation and that the level of phosphorylation remained elevated for up to 2 h in the continued presence of CNTF. Removal of CNTF, however, produced a decrease in the phosphorylation to control levels within 1 h indicating that the stability of the induced tyrosine phosphorylation of 92-kDa STAT3 depended on the continued presence of CNTF. Rapid but transient tyrosine phosphorylation of receptor components and signaling molecules, including STAT proteins, characterizes CNTF and related cytokine responses in a variety of cell lines (14). The functional importance of the stable tyrosine phosphorylation of STAT3 in ciliary ganglion neurons is not clear yet. Cultured embryonic ciliary ganglion neurons are rescued by CNTF, and it is possible that continuous activation of STAT3 is required for CNTF-mediated cell survival. Consistent with this, staurosporine treatment inhibited both CNTF-induced STAT3 tyrosine phosphorylation and CNTF-mediated cell survival.

Conclusion—There are at least three physiological effects of CNTF on cultured embryonic ciliary ganglion neurons: long term survival, increased neuronal growth, and down-regulation of α7-containing nicotinic acetylcholine receptors (20, 24, 41). Induction of STAT3 appears to be specific for the signal transduction pathway induced by CNTF in ciliary ganglion neurons. Identification of the CNTF-inducible 92- and 100-kDa tyrosine-phosphorylated proteins as immunologically identical and functionally similar to STAT3 in ciliary ganglion neurons, which display biologically relevant responses to CNTF/GPA, provides insight into the potential signal transduction mechanism(s) producing these diverse biological effects. The role of the newly identified 100-kDa form of STAT3 in CNTF signal transduction remains to be determined as does the composition of the activated STAT3 transcription complex and its target genes.

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