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CNTF and LIF Act on Neuronal Cells via Shared Signaling Pathways That Involve the IL-6 Signal Transducing Receptor Component gp130

Nancy Y. Ip,* Steven H. Nye,* Teri G. Boulton,* Samuel Davis,* Tetsuya Taga,† Yanping Li,* Susan J. Birren,‡ Kiyoshi Yasukawa,§ Tadamitsu Kishimoto,† David J. Anderson,‖ Neil Stahl,* and George D. Yancopoulos*

*Regeneron Pharmaceuticals, Inc.
777 Old Saw Mill River Road
Tarrytown, New York 10591
†Institute for Molecular and Cellular Biology
Osaka University
1-3, Yamada oka
Suita, Osaka 565
Japan
‡Howard Hughes Medical Institute
‖Division of Biology
California Institute of Technology
Pasadena, California 91125
§Biotechnology Research Laboratory
Tosoh Corporation
Kanagawa
Japan

Summary

Ciliary neurotrophic factor (CNTF) has a variety of actions within the nervous system. While some of the actions of leukemia inhibitory factor (LIF) on neurons resemble those of CNTF, LIF also has broad actions outside of the nervous system that in many cases mimic those of interleukin-6 (IL-6). Comparison of the tyrosine phosphorylations and gene activations induced by CNTF and LIF in neuron cell lines reveals that they are indistinguishable and also very similar to signaling events that characterize LIF and IL-6 responses in hematopoietic cells. We provide a basis for the overlapping actions of these three factors by demonstrating that the shared CNTF and LIF signaling pathways involve the IL-6 signal transducing receptor component gp130. Thus, the receptor system for CNTF is surprisingly unlike those used by the nerve growth factor family of neurotrophic factors, but is instead related to those used by a subclass of hematopoietic cytokines.

Introduction

Ciliary neurotrophic factor (CNTF) was initially identified, purified, and molecularly cloned based on its ability to support the survival of parasympathetic neurons from the chick ciliary ganglion (Adler et al., 1979; Lin et al., 1989; Stockli et al., 1989). Subsequent studies have revealed that CNTF can also enhance the survival of sensory neurons (Skaper and Varon, 1986), motor neurons (Sendtner et al., 1990; Arakawa et al., 1990; Oppenheim et al., 1991), pre-ganglionic sympathetic spinal cord neurons (Blottner et al., 1989), and hippocampal neurons (Ip et al., 1991). In addition to its neuronal survival capabilities, CNTF can inhibit proliferation and enhance cholinergic properties of neuronal precursors from the sympathetic ganglion (Ernsberger et al., 1989), effect cholinergic differentiation of mature sympathetic neurons (Saadat, 1989), and cause the astrocytic differentiation of O-2A glial progenitor cells (Lillien et al., 1988).

Almost nothing is known about the intracellular signaling pathways activated by CNTF, or how these pathways result in the diverse phenotypic responses elicited by CNTF. Potential insights into these transduction pathways come from recent receptor studies. A novel detection scheme revealed that receptors for CNTF were generally restricted to cell lines thought to derive from neuronal precursors, including almost all human neuroepitheliomas and Ewing's sarcomas (a tumor closely related to neuroepithelioma) (Squinto et al., 1990). The ability to detect CNTF receptors, as well as the identification of CNTF receptor–expressing cell lines, allowed for the molecular cloning of a receptor for CNTF (Davis et al., 1991). As expected, this cloned receptor (hereafter referred to as CNTFRα) was almost exclusively expressed within the nervous system. However, it also displayed a number of surprising structural features. First of all, CNTFRα lacks a transmembrane domain and is instead anchored to the membrane via a glycosyl–phosphatidylinositol linkage. Furthermore, it exhibits unexpected homology to one of the two receptor components utilized by a hematopoietic cytokine, interleukin-6 (IL-6). This IL-6 receptor component (hereafter referred to as IL6Rα) binds directly to IL-6 (Yamasaki et al., 1988), but it has a very small intracytoplasmic domain and alone cannot mediate signal transduction (Taga et al., 1989). The second IL-6 receptor component (known as gp130) cannot bind IL-6 directly, but does interact with a complex containing IL-6 bound to the first subunit (Taga et al., 1989; Hibi et al., 1990) to initiate a novel signal transduction pathway that is just beginning to be explored (Nakajima and Wall, 1991; Lord et al., 1991; Murakami et al., 1991). gp130 contains a large intracytoplasmic domain lacking homology with protein kinases and without any known catalytic activities. However, IL-6 treatment results in rapid tyrosine phosphorylations, followed by the activation of a novel protein kinase cascade leading to a characteristic set of immediate early gene responses (including *junB* and *tis*11) (Nakajima and Wall, 1991; Lord et al., 1991; Murakami et al., 1991).

Recent analyses suggest that IL-6 is a distant structural relative of CNTF, as are a number of other hematopoietic cytokines including leukemia inhibitory factor (LIF), granulocyte colony-stimulating factor (G-CSF), and oncostatin M (Bazan, 1991; Rose and Bruce, 1991). LIF, G-CSF, and oncostatin M are all broadly acting factors that, despite having unique growth-regulating activities, share several common actions with IL-6 during hematopoiesis as well as in other processes. For example, all can inhibit the proliferation and induce the differentiation of the murine myeloid leukemia cell line, M1 (Rose and Bruce, 1991).

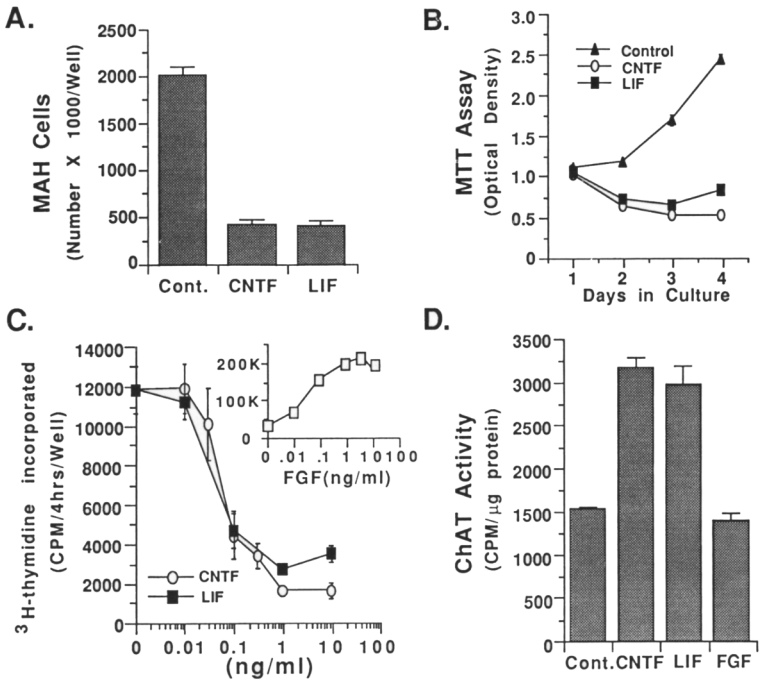

Figure 1. Effects of CNTF, LIF, and FGF on Cell Number, Proliferation Rate, and ChAT Activity of MAH Cells

(A) Effects of CNTF and LIF on cell number quantitated by counting phase-bright cells. MAH cells were plated at a density of 250,000 cells per 35 mm dish, treated with CNTF (10 ng/ml) or LIF (1 ng/ml) for 4 days in culture before counting.

(B) Effects of CNTF and LIF on number of vital cells in MAH cell culture using MTT assay. MAH cells were plated at a density of 6,000 cells per 6 mm well and treated with CNTF (10 ng/ml) or LIF (1 ng/ml) for 1–4 days before MTT assay.

(C) Effects of CNTF, LIF, and FGF on MAH cell proliferation. Various concentrations of CNTF, LIF, and FGF were added to MAH cells. Culture period was continued for 4 days for CNTF and LIF and 3 days for FGF prior to [^{3}H]thymidine incorporation assay. Plating density was at 6,000 cells per 6 mm well for CNTF and LIF and 40,000 cells per 16 mm well for FGF.

(D) Effects of CNTF, LIF, and FGF on ChAT activity in MAH cells. MAH cells were treated with CNTF (10 ng/ml), LIF (1 ng/ml), or FGF (10 ng/ml) for 48 hr, followed by measurement of ChAT activity.

The use of related receptor systems may provide a basis for the similar biological actions of these hematopoietic cytokines; G-CSF and LIF have receptor components that are structurally homologous to gp130 (Fukunaga et al., 1991; Gearing et al., 1991). Furthermore, recent work reveals that LIF induces similar tyrosine phosphorylations and gene activations to IL-6 (Lord et al., 1991). Although similar studies have not been reported for G-CSF or oncostatin M, it seems possible that all of these hematopoietic cytokines utilize gp130-like receptor components to activate similar transduction pathways. The analogies between the cloned CNTF receptor and the IL-6-binding subunit, together with the absence of an intracytoplasmic domain for the glycosyl–phosphatidylinositol-linked CNTFRα, raise the possibility that CNTF also requires a gp130-like second receptor component and may thus activate signaling pathways in neurons that are related to those that cytokines activate in hematopoietic cells (Davis et al., 1991).

Just as IL-6 and its distant relatives elicit common responses in nonneuronal cells, it is known that CNTF and LIF (also known as cholinergic differentiation factor or CDF [Yamamori et al., 1989]) elicit similar responses within some neuronal cells (Rao et al., 1990; Hall and Rao, 1992). Here we identify a neuronal cell line displaying biologically relevant responses to CNTF and find that this line also displays indistinguishable responses to LIF. We explore the signal transduction pathways activated by both CNTF and LIF in this cell line, as well as in other neuronal cell lines, and compare them with those activated by LIF and IL-6 in hematopoietic cell lines. Our studies reveal that CNTF and LIF share signaling pathways that involve the IL-6 signal transducer, gp130, providing a basis for the overlapping actions of these related factors. Thus, while the nerve growth factor family of neurotrophic factors utilizes neuronally restricted receptor tyrosine kinases (the "trks") related to those used by traditional growth factors such as fibroblast growth factor (FGF) and epidermal growth factor, CNTF employs a receptor system that shares components with a subclass of hematopoietic cytokines.

Results

CNTF and LIF Mediate Growth Arrest and Differentiation of MAH Cells

In order to define a neuronal cell line displaying physiologically relevant responses to CNTF, we decided to examine the effects of CNTF on the MAH cell line. The MAH cell line was derived by immortalizing rat sympathoadrenal progenitors with the v-*myc* oncogene (Birren and Anderson, 1990) and might thus represent an immortalized counterpart to CNTF-responsive sympathetic progenitor cells. As with normal sympathetic progenitors, CNTF dramatically inhibited MAH cell proliferation as assessed by cell number (Figure 1A), an MTT (3-(4,5-dimethyl thiazol-2-yl)-2,5-diphenyl tetrazolium bromide) viability assay (Figure 1B), and [^{3}H]thymidine incorporation (Figure 1C). LIF blocked MAH cell proliferation in an indistinguishable manner (Figures 1A, 1B, and 1C), while murine IL-6 had no effect (data not shown). CNTF and LIF displayed very similar dose dependencies on MAH cells, with EC_{50} values of approximately 50 pg/ml (or 2 pM) (Figure 1C), paralleling the CNTF dose dependency required to terminate proliferation of normal sympathetic progenitors (Ernsberger et al., 1989). Unlike either CNTF or LIF, basic FGF acted as a potent mitogen for these

cells (Figure 1C, inset), as shown previously (Birren and Anderson, 1990).

Neither CNTF nor LIF induced neurite extension or other morphological changes characteristic of neuronal differentiation in MAH cells. However, cell cycle analysis showed that CNTF-treated MAH cells were arrested in the G1 phase of the cell cycle (data not shown), reminiscent of many factors that regulate a transition between a proliferative state and cell differentiation (Muggleton-Harris, 1989). CNTF has been shown to enhance cholinergic properties of sympathetic precursors (Ernsberger et al., 1989), and both CNTF and LIF induce cholinergic differentiation of mature sympathetic neurons (Rao et al., 1990). Recently, LIF has been shown to induce choline acetyltransferase (ChAT) mRNA and acetylcholine synthesis in MAH cells (Vandenbergh et al., 1991). In order to pursue the possibility that CNTF and LIF may have a similar differentiative effect on MAH cells, we assayed for the induction of ChAT activity in response to these ligands. As shown in Figure 1D, treatment of MAH cells with CNTF or LIF resulted in similar increases in ChAT activity, in contrast with FGF.

Together, the above data indicate that CNTF and LIF are indistinguishable in their ability to inhibit cell division and enhance cholinergic properties of a sympathoadrenal progenitor cell line. These actions appear quite distinct from those of FGF. In addition to acting as a mitogenic agent for MAH cells, FGF induces neurite outgrowth and initiates neuronal differentiation (but not cholinergic differentiation) of these cells; FGF-induced differentiation may yield a nerve growth factor (NGF)-dependent cell (Birren and Anderson, 1990). Thus, MAH cells apparently provide a very useful model system for dissecting the distinct roles and actions of various factors that can affect the differentiation of neuronal progenitors.

Rapid Tyrosine Phosphorylation of the CLIPs Characterize CNTF and LIF Responses in Neuronal Cells

Although cytokines do not utilize receptors that contain intrinsic tyrosine kinase activity, tyrosine phosphorylation of specific protein substrates is rapidly induced by a variety of different cytokines (reviewed in Miyajima et al., 1992). To determine whether CNTF induces protein tyrosine phosphorylation in responsive cells, and to compare these phosphorylations with those induced by its distant cytokine relatives, we first examined CNTF, LIF, and IL-6 responses in MAH cells as well as in human neuroepithelioma and Ewing's sarcoma cell lines that are known to express CNTFRα (Squinto et al., 1990). As shown in Figure 2, treatment of MAH cells, a Ewing's sarcoma cell line (EW-1), and a neuroepithelioma (SK-N-LO) with CNTF and LIF rapidly induced similar patterns of tyrosine phosphorylation in these lines; in contrast, murine IL-6 did not cause any observable tyrosine phosphorylations in these cells (see below). The CNTF- and LIF-induced phosphorylation patterns were indistinguishable and displayed similar dose dependencies in the different lines examined. The CNTF- and LIF-induced tyrosine phosphorylations included those of three proteins designated as CLIP1 (approximately 190 kd), CLIP2 (approximately 145 kd), and CLIP3 (approximately 75 kd) for CNTF- and

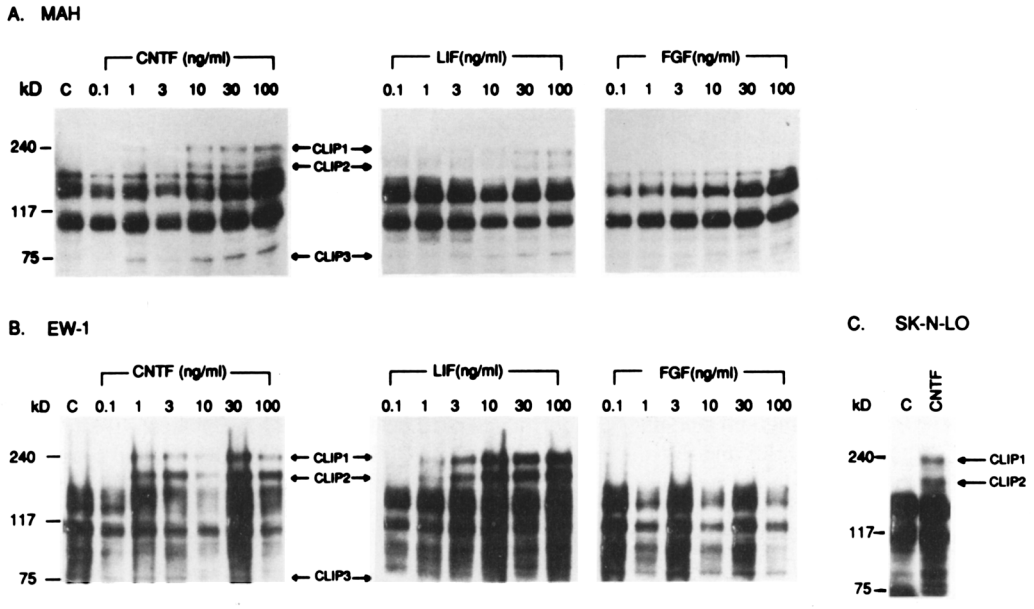

Figure 2. Dose-Dependent Tyrosine Phosphorylation of Proteins in Response to CNTF, LIF, and FGF

Total cell lysates prepared from MAH cells (A), EW-1 cells (B), or SK-N-LO cells (C), following a 5 min treatment with various concentrations (0.1–100 ng/ml) of CNTF, LIF, or FGF, were immunoprecipitated with anti-phosphotyrosine antibody, electrophoresed, and immunoblotted with anti-phosphotyrosine antibody as described in Experimental Procedures. FGF receptor phosphorylation is not detectable in these cells, as is often the case in cells that are expressing low levels of this receptor; FGF-induced tyrosine phosphorylation of lower molecular weight proteins (the ERKs) are, however, detectable (data not shown).

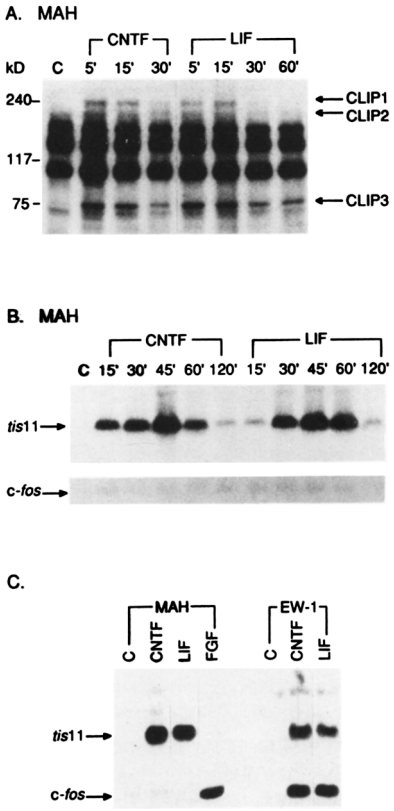

Figure 3. CLIP Phosphorylation Is Rapid and Precedes *tis*11 Gene Induction by CNTF and LIF

(A) CLIP phosphorylation occurs within 5 min of exposure to CNTF and LIF and is down-regulated within 30 min. Total cell lysates from MAH cells treated with 50 ng/ml CNTF or LIF (prepared at various intervals after treatment) were immunoprecipitated and immunoblotted with anti-phosphotyrosine antibody.

(B) Expression of *tis*11 gene, but not of c-*fos*, is rapidly induced following CNTF and LIF treatment. MAH cells were treated with CNTF (10 ng/ml) or LIF (1 ng/ml) for various intervals. Total RNA was then prepared, fractionated by formaldehyde–agarose gel electrophoresis, and hybridized to *tis*11 and c-*fos* DNA probes. The transcript sizes for *tis*11 and c-*fos* were 2.3 kb and 2 kb, respectively.

(C) *tis*11 (but not c-*fos*) is characteristic of CNTF- and LIF-induced response. MAH or EW-1 cells were treated with CNTF (10 ng/ml), LIF (1 ng/ml), or FGF (1 ng/ml) for 30 min. Total RNA was prepared, and expression of *tis*11 and c-*fos* was analyzed by Nothern blotting.

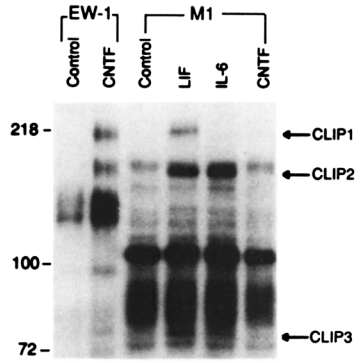

Figure 4. LIF Induces the Same Tyrosine Phosphorylations in a Hematopoietic Cell Line That CNTF and LIF Induce in Neuronal Cell Lines, while IL-6 Induces Only a Subset of These Phosphorylations

EW-1 or M1 cells treated with CNTF (50 ng/ml), LIF (50 ng/ml), or murine IL-6 (100 ng/ml) for 5 min were immunoprecipitated and immunoblotted with anti-phosphotyrosine antibody.

LIF-inducible phosphoproteins; because of difficulty and variability in detection of CLIP3, most subsequent studies focus on CLIP1 and CLIP2. Although both MAH and EW-1 cells are responsive to FGF, the CLIPs are not tyrosine phosphorylated in response to FGF in either cell line (Figure 2). Similarly, the characteristic CLIP phosphorylations did not occur in other cell lines (fibroblasts and PC12 cells) in response to a variety of factors (including NGF, BDNF, NT-3, PDGF, EGF) that utilize receptor tyrosine kinases. Thus, our findings demonstrate that induction of CLIP tyrosine phosphorylation is characteristic of, and relatively specific for, the signal transduction pathways activated by CNTF and LIF.

The Rapid and Transient Tyrosine Phosphorylation of CLIPs Precedes Induction of a Characteristic Immediate Early Response Gene, *tis11*

Signal transduction cascades activated by ligand–receptor interactions often initiate with protein tyrosine phosphorylations and eventually lead to the induction of immediate early response genes (Yarden and Ullrich, 1988). In Figure 3, we have compared the time course of CNTF- and LIF-induced tyrosine phosphorylation with the activation of immediate early gene expression. We examined the expression of one immediate early response gene, *tis*11, which appears to be characteristic of all IL-6-mediated responses, as well as another immediate early response gene, c-*fos*, which is only induced in a subset of IL-6 responses (Nakajima and Wall, 1991).

The induction of tyrosine phosphorylation of the CLIPs by both CNTF and LIF in MAH cells was rapid, occurring within 5 min and significantly decreasing by 30 min (Figure 3A); the kinetics for both factors were similar in EW-1 cells (data not shown). In MAH cells, CNTF and LIF both produced similar inductions in *tis*11 gene expression that followed the induction of CLIP phosphorylation. Maximal activation occurred at 45 min and returned to control levels by 120 min (Figure 3B); similar gene activation kinetics for *tis*11 were observed in EW-1 cells (data not shown). No induction of c-*fos* expression was observed in MAH cells with either CNTF or LIF (Figure 3B), although these factors did induce c-*fos* as well as *tis*11 in EW-1 cells (Figure 3C). In contrast with CNTF and LIF, bFGF induced c-*fos* gene expression in the absence of *tis*11 gene induction in MAH cells (Figure 3C).

CNTF and LIF Trigger Kinase Cascades in Neuronal Cell Lines Similar to Those Used by LIF and IL-6 in Hematopoietic Cell Lines

Our results suggest that rapid phosphorylation of the CLIPs, followed by the induction of *tis*11 gene expression, characterize both CNTF and LIF responses in neuronal cell lines. A direct comparison of these signaling events

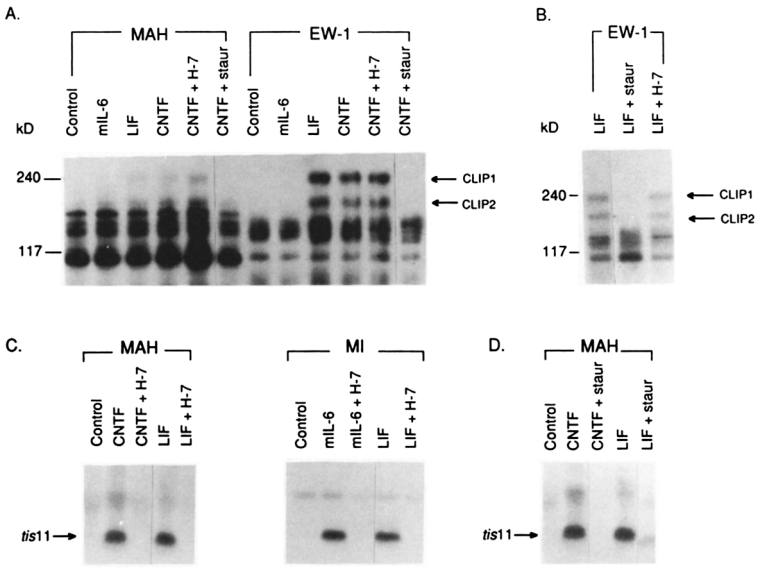

Figure 5. Effects of Protein Kinase Inhibitors on CLIP Phosphorylation and *tis*11 Gene Expression Verify That CNTF, LIF, and IL-6 Utilize Similar Signal Transduction Pathway

(A and B) Staurosporine, but not H-7, blocks CLIP phosphorylation induced by both CNTF (A) or LIF (B). Cells were treated with H-7 (40 μg/ml) or staurosporine (100 nM) for 15 min prior to addition of CNTF (50 ng/ml), LIF (50 ng/ml), or murine IL-6 (100 ng/ml). Total cell lysates were immunoprecipitated and immunoblotted with anti-phosphotyrosine antibody.

(C) H-7 inhibits *tis*11 gene induction by CNTF and LIF in MAH cells, or by LIF and IL-6 in M1 cells. Cells were treated with H-7 (40 μg/ml) for 30 min prior to addition of CNTF (50 ng/ml), LIF (50 ng/ml), or murine IL-6 (100 ng/ml). Total RNA was prepared and subjected to Northern analysis using a *tis*11 probe.

(D) Staurosporine inhibits *tis*11 gene induction by CNTF and LIF in MAH cells. Cells were treated with staurosporine (100 nM) for 30 min prior to addition of CNTF (50 ng/ml) or LIF (50 ng/ml). Total RNA was prepared and subjected to Northern analysis using a *tis*11 probe.

with those triggered by LIF and IL-6 in hematopoietic cells reveals striking similarities as well as differences (Figure 4). LIF induces the tyrosine phosphorylation of proteins identical in size to CLIP1, CLIP2, and CLIP3 in the M1 myeloid progenitor cell line, whereas IL-6 induces the tyrosine phosphorylation of only two of these proteins, apparently corresponding to CLIP2 and CLIP3. The phosphorylation of CLIP1 by LIF but not IL-6 represents the first detectable difference between these two pathways; intriguingly, CLIP1 is similar in size to that reported for the recently cloned LIF receptor (see discussion). CNTF did not induce any detectable tyrosine phosphorylation in M1 cells (Figure 4), which lack the CNTFRα (see below).

Specific phosphorylation events can be distinguished using different protein kinase inhibitors. We first utilized the protein kinase inhibitor H-7 to determine whether the kinase cascades leading to *tis*11 activation are similar for CNTF, LIF, and IL-6; H-7 was used because it specifically blocks a downstream kinase required for gene inductions by IL-6 and LIF in hematopoietic cells without affecting the initial tyrosine phosphorylation events (Nakajima and Wall, 1991; Lord et al., 1991). While H-7 did not block CNTF (Figure 5A), and LIF (Figure 5B) induced tyrosine phosphorylation events in either MAH or EW-1 cells, H-7 did block *tis*11 induction by either CNTF or LIF in MAH cells, just as it blocked *tis*11 induction by IL-6 and LIF in M1 cells (Figure 5C).

Examination of other kinase inhibitors revealed that a relatively specific tyrosine kinase inhibitor known as staurosporine (S. H. N., S. P. Squinto, D. J. Glass, P. Hantzopoulos, M. J. Macchi, N. S. Lindsay, N. Y. I., and G. D. Y., unpublished data) could very effectively prevent CLIP phosphorylation by either CNTF or LIF (Figures 5A and 5B). Providing evidence that CLIP phosphorylation not only precedes, but is required for subsequent gene inductions, staurosporine also prevented *tis*11 gene induction by CNTF or LIF (Figure 5D).

Thus, a direct comparison of phosphorylation and gene induction events, together with the use of protein kinase inhibitors, demonstrates that the signaling pathway activated by CNTF and LIF in neuronal cell lines appears very similar to that utilized by LIF in hematopoietic cells. As would be expected if CNTF and LIF share signal transducing components, down-regulation of CLIP1 and CLIP2 phosphorylation due to pretreatment with CNTF or LIF could not be overcome by subsequent addition of the other factor (Figure 6A). Thus, CNTF and LIF seem to share an identical signaling pathway that is distinguishable from (i.e., with reference to CLIP1), but shares many of the novel features of, the IL-6-activated pathway in hematopoietic cells.

CLIP1 and CLIP2 Are Cell Surface Proteins That Are Part of Receptor Complexes

To determine whether the CLIPs are expressed on the cell surface, we utilized an assay that specifically results in the biotinylation of cell surface proteins (Stahl et al., 1990). This assay revealed that CLIP1 and CLIP2 did indeed express extracellular domains that could be biotinylated (Figure 6B); the surface location of CLIP1 was also consistent with the finding that its apparent size decreased upon peptide N-glycosidase F treatment (data not shown).

The finding that CLIP1 and CLIP2 are cell surface proteins that rapidly become tyrosine phosphorylated in response to either CNTF or LIF suggested that these proteins might be part of the receptor complexes utilized by these factors. To explore this possibility, EW-1 cells were incubated with biotinylated CNTF (bCNTF), lysed in nonionic detergent, and then precipitated with streptavidin–agarose to purify receptor proteins that might be associ-

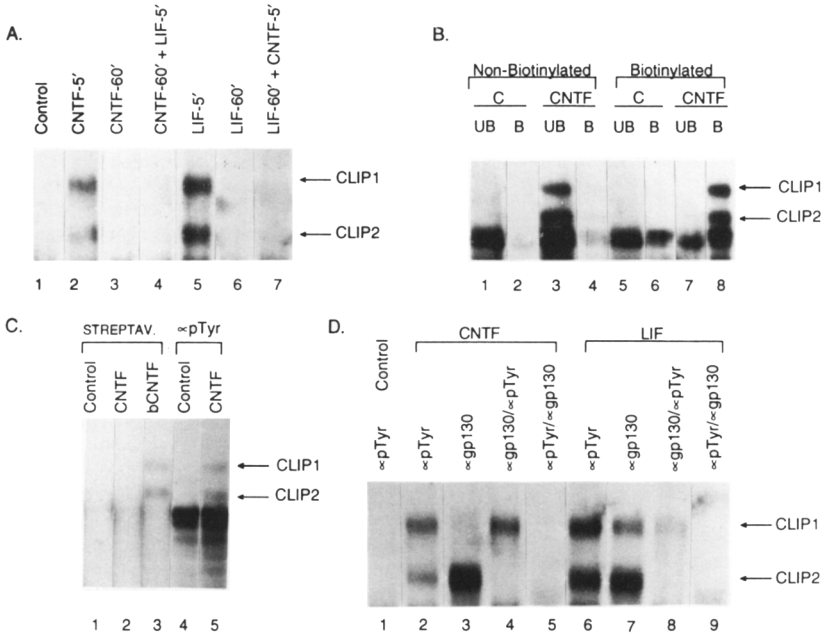

Figure 6. CLIP1 and CLIP2 Are Coregulated by CNTF and LIF, Are on the Cell Surface, and Are Part of the CNTF Receptor Complex; One of the CLIPs (CLIP2) Is gp130

(A) CLIP1 and CLIP2 are coregulated by CNTF and LIF. EW-1 cells were treated for 5 or 60 min with either CNTF (50 ng/ml) or LIF (50 ng/ml), as indicated; after the 60 min timepoints, either additional CNTF (lanes 3 and 7) or LIF (lanes 4 and 6) were added to the cells for 5 additional min. Total cell lysates were then immunoprecipitated and immunoblotted with anti-phosphotyrosine antibody.

(B) Biotinylation assay reveals that CLIP1 and CLIP2 are on the cell surface. EW-1 cells were surface biotinylated as described in Experimental Procedures. The figure shows the anti-phosphotyrosine immunoblot for control (C) or CNTF-stimulated (CNTF) cells that were subsequently biotinylated or left nonbiotinylated before separation into unbound (UB) or bound (B) fractions on streptavidin–agarose.

(C) CLIP1 and CLIP2/gp130 associate with biotinylated CNTF and are thus part of the CNTF receptor complex. EW-1 cells were treated with no factor (lane 1 and 4), CNTF (lane 2 and 5), or biotinylated CNTF (bCNTF, lane 3) for 5 min, solubilized with lysis buffer containing 1% NP40, and then incubated with streptavidin–agarose or anti-phosphotyrosine antibodies conjugated to agarose. Lanes 1 to 5 compare the anti-phosphotyrosine immunoblot of proteins bound to streptavidin–agarose beads (lanes 1 to 3) with proteins immunoprecipitated with anti-phosphotyrosine antibodies (lanes 4 and 5).

(D) CLIP2 is gp130. The figure shows the anti-phosphotyrosine immunoblot of lysates from control (C) or CNTF/LIF-stimulated EW-1 cells that were immunoprecipitated with the anti-phosphotyrosine antibody (αpTyr) or the gp130-specific antibody (αgp130). The immunoprecipitating antibodies were either used individually or in sequential manner, as indicated.

ated with the bCNTF. Immunoblotting of these precipitates with anti-phosphotyrosine revealed that they contained tyrosine phosphorylated proteins that precisely comigrated with CLIP1 and CLIP2 (Figure 6C); no other tyrosine phosphorylated proteins could be detectably coprecipitated with bCNTF. These results demonstrate that CLIP1 and CLIP2 are indeed part of the CNTF receptor complex. Although the CNTFRα component can also be complexed with bCNTF (data not shown), it would not be detected by this assay because of its lack of tyrosine phosphorylation.

CNTF and LIF Share the IL-6 Signal Transducing Receptor Component gp130

Recent observations demonstrated that the IL-6 signal transducing receptor component gp130 is tyrosine phosphorylated in response to IL-6 in hematopoietic cell lines (Murakami et al., 1991). The similarities of the CNTF/LIF pathways to those activated by IL-6, together with the observed phosphorylation of CLIP2 in response to IL-6 stimulation of M1 myeloid cells, raised the intriguing possibility that the apparent CNTF/LIF receptor component CLIP2 (which we estimated to have a molecular weight of approximately 145 kd) is indeed gp130, and thus that all three of these factors activate a shared signal transducing receptor component. This possibility was first tested by using a monoclonal antibody (AM64) specific for human gp130 (Hibi et al., 1990) in concert with a human cell line (EW-1) responsive to both CNTF and LIF; AM64 apparently does not bind any gp130-related proteins, nor does it even identify gp130 from rodent species. This gp130-specific antibody could be used to deplete CLIP2 completely from extracts of CNTF- or LIF-induced EW-1 cells (compare lanes 4 and 8 with lanes 2 and 6 in Figure 6D); furthermore, the immunoprecipitates obtained using the AM64 antibody contained CLIP2 (compare lanes 3 and 7 with lanes 2 and 6 in Figure 6D). From these data, we infer that CLIP2 is gp130, and that gp130 is tyrosine phosphorylated in response to both CNTF and LIF. Consistent with the data presented in Figure 6C, CLIP1 partially coprecipitates with gp130/CLIP2 when using the AM64 antibody (lanes 3 and 7 in Figure 6D), verifying that these two molecules are part of the same receptor complexes; varying the lysis conditions affects the amount of CLIP1 coprecipitating

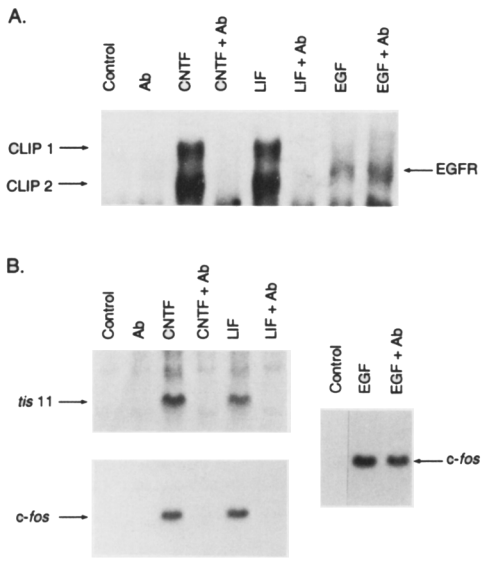

Figure 7. gp130-Specific Blocking Antibodies Prevent CNTF- and LIF-Induced Signaling Responses, but Not Responses Due to an Unrelated Growth Factor, EGF

(A) gp130-specific blocking antibodies prevent CNTF- and LIF-induced tyrosine phosphorylation of CLIP1 and gp130/CLIP2 in EW-1 cells, but not EGF-induced tyrosine phosphorylation of its receptor; EGF was used instead of FGF because of its ability to detect EGF receptor autophosphorylation. Total cell lysates from EW-1 cells treated with CNTF, LIF, or EGF (in the absence or presence (+ Ab) of the blocking antibody), were immunoprecipitated and immunoblotted with anti-phosphotyrosine-specific antibodies.

(B) CNTF- and LIF-induced *tis*11 and *c-fos* gene responses are prevented by pre-treatment with gp130-specific blocking antibodies, while EGF-induced *c-fos* expression is not affected. Total RNA prepared from EW-1 cells treated with the indicated factors (in the presence or absence of the blocking antibody) was fractionated by formaldehyde–agarose gel electrophoresis and hybridized to *tis*11 and *c-fos* DNA probes.

with gp130/CLIP2 in response to CNTF and LIF treatment (data not shown).

Additional monoclonal antibodies specific for human gp130 have recently been selected based on their ability to block IL-6-mediated responses (T. T., K. Y., and T. K., unpublished data). Pretreatment of EW-1 cells with these gp130-specific blocking antibodies completely prevented both CNTF- and LIF-induced tyrosine phosphorylations and subsequent gene responses (Figures 7A and 7B). In contrast, these antibodies did not affect the ability of an unrelated growth factor (epidermal growth factor [EGF]) to induce receptor tyrosine phosphorylation or subsequent gene responses in EW-1 cells (Figures 7A and 7B). These studies using gp130-specific blocking antibodies not only support the finding that CLIP2 is gp130, but further demonstrate that gp130 is absolutely required for both CNTF and LIF signaling processes.

Ubiquitous Expression of gp130 Contrasts with Restricted Expression of CNTFRα

It had previously been speculated that gp130 might function as a transducer for factors other than IL-6 based on the finding that gp130 transcripts were much more

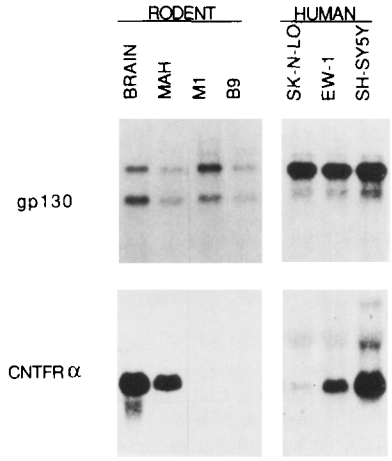

Figure 8. Ubiquitous Distribution of gp130 mRNA Contrasts with Restricted Neuronal Distribution of CNTFRα mRNA

Total RNA was prepared from the indicated lines and subjected to Northern blot analysis using either a human gp130 cDNA probe (top panels) or a rat CNTFRα cDNA probe (bottom panels); the weaker hybridization to the rodent lines with the gp130 probe is due to poor cross-species hybridization. SH-SY5Y, neuroblastoma; EW-1, Ewing's sarcoma; SK-N-LO, neuroepithelioma; MAH, sympathoadrenal progenitor; M1, myeloid progenitor; B9, IL-6-dependent B cell hybridoma that does not respond to CNTF.

widely distributed than those for IL6Rα (Hibi et al., 1990). Consistent with this notion and our finding that gp130 is shared by the CNTF and LIF signaling systems, we find that gp130 transcripts are expressed in both hematopoietic lines responsive to IL-6 (but not CNTF) (Figure 8, note M1 and B9 cell lines), as well as in adult brain and neuronal lines responsive to CNTF and LIF (but not IL-6) (Figure 8, note MAH, EW-1, SK-N-LO, and SH-SY5Y cell lines). In contrast, CNTFRα mRNA displays a restricted distribution and is expressed only in the brain and in neuronal lines responsive to CNTF (Figure 8).

Discussion

In the case of IL-6, a complex between IL-6 and its a receptor component binds gp130, which then somehow activates the signal transduction process (Taga et al., 1989; Hibi et al., 1990). The ability of gp130 to transduce functional signals correlates with its ability to be phosphorylated on tyrosine (Murakami et al., 1991). Here we have identified cell lines that allow for comparison of responses to CNTF and LIF, distant structural relatives of IL-6. Strikingly, the CNTF-responsive neuronal cell lines examined displayed indistinguishable phenotypic and biochemical responses to LIF; in contrast, LIF-responsive hematopoietic cells did not respond to CNTF. CNTF and LIF responses in neuronal cells appear to initiate with the tyrosine phosphorylation of the three CLIPs, at least two of which (CLIP1 and CLIP2) are cell surface proteins that can interact and form part of the receptor complexes. The CLIP phosphorylations precede and, based on both kinase inhibitor and blocking antibody studies, apparently reflect activation events required for subsequent characteristic

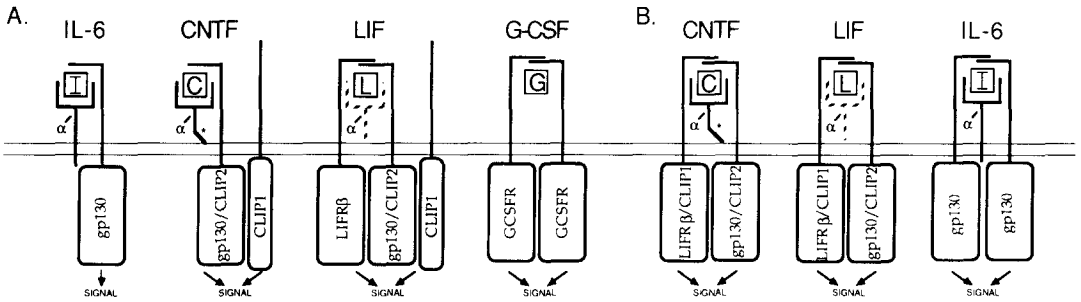

Figure 9. Schematic Models of G-CSF, IL-6, CNTF, and LIF Receptor Complexes

(A) Model depicting known components of indicated cytokine receptor complexes.

(B) Revised "unified" models of CNTF and LIF receptor complexes assuming that CLIP1 is LIFRβ and that gp130 functions as a dimer in the IL-6 receptor complex. In the model presented in (B), CNTFRα is all that is required to convert a functional LIF receptor complex into a functional CNTF receptor complex. Factors are represented as squares; α subunits are known to exist for the IL-6 and CNTF receptor complexes and are thus depicted with solid lines (asterisk adjacent to CNTFRα–membrane junction indicates glycosyl–phosphatidylinositol linkage), while potential LIFRα component is indicated by a dashed line. Although depicted as membrane bound, α subunits may also function as soluble cofactors (see text).

gene inductions. LIF and CNTF display parallel dose responses, time courses, and inhibitor profiles with respect to these phosphorylations and gene inductions, and prior treatment with either factor will down-regulate responses to the other. Not only are the CNTF- and LIF-induced signaling events essentially indistinguishable in neuronal cells, but they appear identical to those induced by LIF in hematopoietic cells. These events are also very similar to those induced by IL-6 in hematopoietic cells, except that CLIP1 phosphorylation is specifically characteristic of CNTF and LIF responses. We provide a basis for the similarities in the CNTF, LIF, and IL-6 signaling pathways by demonstrating that one of the CLIPs (CLIP2) is the IL-6 signal transducer, gp130.

Our findings raise many questions concerning the interactions of the various CNTF and LIF receptor components with gp130/CLIP2. CNTF can bind directly to the IL6Rα-related CNTFRα (Davis et al., 1991) that, based on our data and by analogy to the IL-6 system (Figure 9A), then presumably interacts with gp130/CLIP2. However, the CNTF receptor complex apparently also includes another cell surface protein, CLIP1, that is tyrosine phosphorylated in response to CNTF and can directly interact with gp130/CLIP2 (Figure 9A). LIF is known to bind a recently cloned gp130-related receptor component with a molecular weight of approximately 190 kd (hereafter referred to as LIFRβ), and the existence of a LIF-receptor α component (hereafter referred to as LIFRα) has also been proposed (Gearing et al., 1991). Our data indicate that the LIF receptor complex also includes CLIP1 and gp130/CLIP2 (Figure 9A). Finally, the receptor complex for IL-6/CNTF/LIF-related G-CSF is apparently a homodimer of the gp130-related G-CSF receptor (Fukunaga et al., 1991) (Figure 9A).

Although the receptor complexes portrayed in Figure 9A mediate binding to structurally related ligands, as depicted they are unsatisfyingly different. It is possible to propose more "uniform" receptor models, however, if one considers the possibility that CLIP1, which is similar in size to LIFRβ, is indeed LIFRβ (Figure 9B). Thus, the CNTF and LIF receptor complexes would each utilize two different gp130-like components, LIFRβ/CLIP1 as well as gp130/CLIP2 itself. These two "β" components would directly interact based on our coprecipitation data, and they would both be inducibly phosphorylated on tyrosine. Supporting such a receptor structure, recent crosslinking data (Godard et al., 1992) reveal that LIF can be bound to two distinct proteins with sizes that would correspond to those of LIFRβ/CLIP1 and gp130/CLIP2. The involvement of two β components in the CNTF and LIF receptor complexes would be reminiscent of the G-CSF receptor structure (Figure 9A) and raises the possibility that the IL-6 receptor complex may also involve a homodimer of gp130 (Figure 9B). In fact, it may be that β subunit dimerization and/or aggregation leads to activation of the signaling process, as proposed for receptor tyrosine kinases and some cytokine receptors (Aaronson, 1991; De Vos et al., 1992).

In the model presented in Figure 9B, the α receptor components would act to modulate the binding of the factors to the β components and thus be responsible for conferring ligand specificity upon the shared transducing machinery. Cross-linking data (Godard et al., 1992) might suggest that for LIF, as with G-CSF, such a component may not be required. Thus, the CNTFRα component would be all that is required to convert a functional LIF receptor into a functional CNTF receptor. The latter possibility, together with the restricted expression of CNTFRα to the nervous system (Davis et al., 1991), could explain why all CNTF-responsive neuronal cells also respond identically to LIF (see above; also Rao et al., 1990), whereas LIF-responsive cells outside of the nervous system do not respond to CNTF. It remains to be determined whether LIF actions on CNTF receptor–bearing cells are physiologically relevant, or whether they simply reflect the fact that the two proteins comprising the LIF receptor complex are contained within the larger CNTF receptor complex.

Interestingly, the α components for IL-6 or CNTF do not have to be membrane bound in order to interact with their transducing components (Taga et al., 1989; S. D. and G. D. Y., unpublished data). Thus, complexes containing

these factors together with soluble forms of their α receptors may act as heterodimeric factors for cells that are not capable of responding to the factor alone (because they do not express the appropriate α receptor), but which do express the appropriate transducing components. The possibility that such heterodimeric complexes actually operate as soluble factors in vivo is supported by the homology between the α receptor components and one of the two subunits of natural killer cell stimulatory factor, a normally occurring heterodimeric factor (Gearing and Cosman, 1991). Furthermore, the unusual and readily cleavable glycosyl–phosphatidylinositol linkage of the CNTFRα to the cell surface points toward a role for regulated release of this receptor component (Davis et al., 1991).

While the receptor models presented above need further experimental verification, they clearly have relevant precedents. A plethora of G protein–coupled receptors similarly interact with a small number of signal transducing heterotrimeric G proteins, allowing a vast array of different signals (e.g., neurotransmitters, polypeptide hormones, and odorants) to converge on a relatively modest number of signaling pathways (Gilman, 1987). More directly relevant to the gp130-coupled receptor systems are those of IL-3, IL-5, and GM-CSF. The overlapping activities and similar tyrosine phosphorylations induced by IL-3, IL-5, and GM-CSF led to the finding that these factors use distinct α receptor components but share β components (reviewed in Nicola and Metcalf, 1991; Miyajima et al., 1992). Once again, the α receptor components are primarily involved in binding the factors, but lack extensive cytoplasmic domains and thus do not appear to have signal transducing capabilities. The shared β subunits appear to be required for high affinity binding and are responsible for initiating signal transduction events that involve tyrosine phosphorylations. As with gp130 (and presumably LIFRβ/CLIP1), the β subunits are themselves tyrosine phosphorylated, but do not appear to have inherent kinase activity. Although little is known about the mechanisms by which these β subunits are tyrosine phosphorylated, the multicomponent IL-2 receptor also utilizes a β subunit (IL2Rβ) that is responsible for high affinity binding and signal transduction, and this β chain is tyrosine phosphorylated by a src-like tyrosine kinase (lck) with which it physically associates (reviewed in Miyajima et al., 1992). Interestingly, CLIP phosphorylation and IL-2-induced lck phosphorylation display similar susceptibility profiles to kinase inhibitors (Hatakeyama et al., 1991). That is, both phosphorylations are susceptible to staurosporine but not to H-7, suggesting that similar tyrosine kinases may be involved; CLIP3 may be a candidate for such a src-related kinase.

It is perhaps surprising that the receptor system for CNTF is so unlike the "trk" receptor kinases used by the nerve growth factor family of neurotrophic factors, but instead shares components with the receptor complexes for a subclass of hematopoietic cytokines. On the other hand, there are certainly striking parallels between the growth inhibitory, differentiative, and survival roles played by the hematopoietic cytokines in the immune system and the actions displayed by CNTF within the nervous system. The CNTF receptor system differs most dramatically from those of the hematopoietic cytokines in that its α receptor component is expressed almost exclusively within the nervous system (Davis et al., 1991; N. Y. I. and G. D. Y., unpublished data); it is only in this respect that the CNTFRα component more closely resembles the "trk" receptors for the nerve growth factor family. Our studies suggest that the shared, and generally expressed, signal transducing receptor components provide the basis for the striking parallels in the type of activities displayed by the hematopoietic cytokines and CNTF, but that the limited distribution of CNTFRα is responsible for restricting CNTF actions to the nervous system. This restriction contrasts with the broad actions of the cytokines related to CNTF and raises the possibility that additional related cytokines displaying a very restricted range of actions may exist. From an evolutionary standpoint, it is fascinating to consider whether the broadly acting cytokines gave rise to the more specifically acting CNTF, or whether the reverse occurred.

Identification of the MAH cell line provides a neuronal precursor cell line that displays physiologically relevant responses to CNTF and LIF as well as to factors using unrelated receptor systems, such as FGF and NGF. Use of the MAH cell line should contribute to the understanding of how different factors, utilizing distinct signaling pathways, can interact to affect the growth and differentiation of neuronal progenitor cells. Contrasting the responses of MAH cells and hematopoietic cell lines to the cytokines should also provide insight into the mechanisms by which distinct cellular contexts alter the perception and interpretation of a very similar initial signal.

Experimental Procedures

Reagents

Preparation and purification of recombinant rat CNTF used in this study has been previously described (Masiakowski et. al., 1991). Murine IL-6 was purchased from Upstate Biotechnology, Inc.; recombinant human LIF was from Amgen Biologicals. Basic FGF purified from bovine brain was purchased from R & D Systems, and NGF was purified from mouse submaxillary gland (Darling and Shooter, 1983). Protein kinase inhibitors used include H-7 [1-(5-isoquinolinylsulfonyl)-2-methylpiperazine dihydrochloride], Seikagaku Kogyo Co.) and staurosporine (Kamiya Biomed. Co.). Anti-phosphotyrosine monoclonal antibody conjugated to agarose beads was purchased from Upstate Biotechnology, Inc.

Cell Culture

MAH cells were maintained in culture as previously described (Birren and Anderson, 1990). In brief, cells were plated onto dishes precoated with poly-D-lysine (100 μg/ml) and laminin (10 μg/ml) at a density of 6,000 per 6 mm well, or 40,000 per 16 mm well. Medium used was modified L15-CO_2 medium supplemented with 10% fetal bovine serum and dexamethasone (5 μM). IARC-EW-1 (Ewing sarcoma cells), SK-N-LO (neuroepithelioma cells), and SH-SY5Y (neuroblastoma) cells were cultured in RPMI 1640 medium with 10% fetal bovine serum supplemented with 2 mM L-glutamine and 100 U/ml penicillin and streptomycin. M1 myeloid progenitor cells were cultured as previously described (Lord et al., 1991).

MTT Assay, [^{3}H]Thymidine Incorporation Assay, and ChAT Assay

MAH cells were treated with factors for various periods of time, prior to the addition of MTT dye (final concentration of 0.5 mg/ml). Incubation was continued for 8 hr, and dimethyl sulfoxide was added to solubilize the dye product taken up by vital cells. The optical density at 570–

650 nm was quantitated using the Flow Titretek multiscan apparatus (Manthorpe et al., 1986). For [^{3}H]thymidine incorporation assay, cells were treated with various factors for different periods of time, and [^{3}H]thymidine (NEN-NET-027E purchased from New England Nuclear) was added at a final concentration of 1 μCi/ml and incubated for 4 hr at 37°C. Cells were then washed three times with phosphate-buffered saline (PBS), lysed with sodium hydroxide (0.5 N) for 2 hr at room temperature, and [^{3}H]DNA was counted. ChAT assays were performed as previously described (Fonnum, 1975). In brief, cells were treated with various factors, washed with ice-cold PBS, then ChAT harvest buffer (containing 20mM Tris–HCl [pH 8.6] and 0.1% Triton X-100) was added and incubated for 15 min on ice. Cell extracts were then incubated for 60 min at 37°C with a reaction mixture containing 11 mM choline chloride, 0.2 mM ^{14}C–acetyl coenzyme A, 0.14 mM physostigmine, 300 mM sodium chloride, 50 mM sodium phosphate, and 20 mM EDTA. An aliquot of this mixture was mixed with scintillation fluid containing acetonitrile–teraphenylboron (5 mg/ml) and counted.

RNA Isolation and Analysis

Cells were plated at a density of 5 × 10^6 cells on 100 mm dishes and treated with factors for various periods of time. Total RNA was prepared by the guanidinium thiocyanate method as described previously (Chomczynski and Sacci, 1987). Ten micrograms of RNA was electrophoresed on a formaldehyde–agarose gel, transferred to a nylon membrane (MSI), and hybridized to ^{32}P-probes labeled by random oligo-priming (Stratagene). The probes used included *tis*11 (2.3 kb EcoRI fragment), *c-fos* (1kb PstI fragment), human gp130 (3.0 kb EcoRI–SpeI fragment), and rat CNTFRα (800 bp PstI fragment) cDNA fragments.

Protein Isolation, Immunoprecipitation, Immunoblotting, and gp130-Blocking Antibodies

For the detection of protein tyrosine phosphorylation, cells were starved for 60 min in serum-free, defined medium, treated with various factors for 5 min, and protein lysates prepared with RIPA buffer (supplemented with proteinase and phosphatase inhibitors) as previously described (Glass et al., 1991). To prepare total protein samples, protein loading dye was added directly to the RIPA lysate supernatants and boiled for 3 min at 90°C. For immunoprecipitations, supernatants from the RIPA lysates were precipitated overnight at 4°C with either anti-phosphotyrosine antibodies conjugated to agarose (UBI), or the human gp130-specific mouse monoclonal antibody AM64 (Hibi et al., 1990) followed by a goat anti-mouse IgG antibody conjugated to agarose (purchased from Sigma). Proteins were eluted from agarose beads with 200 μl of protein-loading dye and boiled for 3 min. Total protein samples or immunoprecipitate (each equivalent to approximately one million [EW-1, MAH, SK-N-LO] or ten million [M1] cells) were electrophoresed on either 7.5% or 10% SDS–polyacrylamide gels, immunoblotted with anti-phosphotyrosine antibodies as previously described (Glass et al., 1991), and specific proteins detected with ^{125}I-labeled goat anti-mouse polyclonal antibody (1 μl of antibody labeled to a specific activity of 4.91 μCi/μg [purchased from New England Nuclear] was added to each milliliter of buffer). CLIP molecular weights were estimated from semi-logarithmic plots against pre-stained standards (Bethesda Research Laboratories).

The gp130-blocking experiments were performed using either a mixture of three anti-human gp130 mouse monoclonal antibodies (GPX7, GPX22, and GPZ35), or just the GPX22 antibody alone, at a total antibody concentration of 2 μg/ml, applied 60 min prior to ligand challenge. Results with the GPX22 antibody alone are presented in Figure 7; the mixture of antibodies yielded identical results.

Cell Surface Biotinylation Assay

Following a 5 min incubation with LIF or CNTF to induce CLIP phosphorylation, cells were washed in 5 ml of PBS supplemented with 1 mM orthovanadate (PBSV), then incubated for 10 min on ice in PBSV containing 1 mg/ml NHS-SS-biotin (3-sulfosuccinimido 3-[2-(biotinamido) ethyl] dithioproprionate; Pierce), a membrane-impermeant reagent. The plates of cells were then washed with Tris-buffered saline containing orthovanadate and lysed with RIPA buffer as described above. Lysates were precipitated with immobilized anti-phosphotyrosine antibody as described, then bound phosphoproteins were removed from the beads by boiling for 5 min in 50 mM Tris (pH 8.2) containing 1% SDS. Biotinylated proteins were precipitated from this solution by incubation for 1 hr with 20 μl of streptavidin–agarose (Pierce). The supernatant containing the nonbiotinylated proteins was subjected to SDS–polyacrylamide gel electrophoresis after the addition of sample buffer. The beads containing biotinylated proteins were washed once in the binding buffer, then the bound biotinylated proteins were eluted from the beads by boiling for 5 min in 2 × SDS–polyacrylamide gel electrophoresis sample buffer containing 10% β-mercaptoethanol. Anti-phosphotyrosine immunoblotting on these samples was performed as described above.

CNTF Biotinylation and Use in Precipitation of Proteins in Receptor Complex

CNTF (150–400 μg in PBS, [pH 7.4]) was incubated with a 5-fold molar excess of NHS-LC-biotin (Pierce, typically dissolved to 5 mg/ml in PBS immediately before use) in a volume of 500 μl or less for 2 hours at 4°C. The reaction was quenched by the addition of 50 μl of 3 M Tris (pH 8.2), for approximately 15 min. Biotinylated mono and dimeric CNTF were purified and separated from excess biotinylation reagent by chromatography on a Superdex 75 HR 10/30 column (Pharmacia) using a Pharmacia FPLC system pumping PBS at a flow rate of 1 ml/min. Dimer and monomer bCNTF were eluted at times of 10 and 12 min, respectively. Protein concentration and recovery was determined by optical density readings at 280 nm using an extinction coefficent of $\varepsilon = 2.05 \times 10^4\,cm^{-1}\,M^{-1}$. The monomeric biotinylated CNTF was then added (at a concentration of 50 ng/ml) to cells for 5 min, after which the cells were washed twice in lysis buffer (TBS containing 50 mM sodium fluoride, 1 mM vanadate, 5 mM benzamidine, 1 mM EDTA, 10 μg/ml leupeptin, 10 μg/ml aprotinin, 1 mM phenylmethylsulfonyl fluoride) and then lysed in the same buffer supplemented with 1% NP40 (500 μl were used per 100 mm plate of cells). The lysates were then precleared for 40 min at 4°C using 50 μl of goat anti-rabbit antibodies conjugated to agarose, and then incubated overnight with 50 μl of streptavidin conjugated to agarose. The pelleted precipitate was then resuspended in sample buffer and gel electrophoresed.

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Notes Added In Proof

While this manuscript was under review, Gearing et al. (Science *255*, 1434–1437) reported that coexpression of gp130 and LIFRβ produces

high affinity binding sites for LIF, which is consistent with the model presented in this paper.

The data referred to as "S. H. N. et al., unpublished data" can now be updated: Nye, S. H., Squinto, S. P., Glass, D. J., Hantzopoulos, P., Macchi, M. J., Lindsay, N. S., Ip, N. Y., and Yancopoulos, G. D. (1992). K-252a and staurosporine selectively block autophosphorylation of neurotrophin receptors and neurotrophin-mediated responses. Mol. Biol. Cell, in press.