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Signaling Pathways Recruited by the Cardiotrophin-like Cytokine/Cytokine-like Factor-1 Composite Cytokine

SPECIFIC REQUIREMENT OF THE MEMBRANE-BOUND FORM OF CILIARY NEUROTROPHIC FACTOR RECEPTOR α COMPONENT*

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Ciliary neurotrophic factor (CNTF) is a cytokine supporting the differentiation and survival of a number of neural cell types. Its receptor complex consists of a ligand-binding component, CNTF receptor (CNTFR), associated with two signaling receptor components, gp130 and leukemia inhibitory factor receptor (LIFR). Striking phenotypic differences between CNTF- and CNTFR-deficient mice suggest that CNTFR serves as a receptor for a second developmentally important ligand. We recently demonstrated that cardiotrophin-like cytokine (CLC) associates with the soluble orphan receptor cytokine-like factor-1 (CLF) to form a heterodimeric cytokine that displayed activities only on cells expressing the tripartite CNTF receptor on their surface. In this present study we examined the membrane binding of the CLC/CLF composite cytokine and observed a preferential interaction of the cytokine with the CNTFR subunit. Signaling pathways recruited by the CLC/CLF complex in human neuroblastoma cell lines were also analyzed in detail. The results obtained showed an activation of Janus kinases (JAK1, JAK2, and TYK2) leading to a tyrosine phosphorylation of the gp130 and LIFR. The phosphorylated signaling receptors served in turn as docking proteins for signal transducing molecules such as STAT3 and SHP-2. *In vitro* analysis revealed that the gp130-LIFR pathway could also stimulate the phosphatidylinositol 3-kinase and the mitogen-activated protein kinase pathways. In contrast to that reported before for CNTF, soluble CNTFR failed to promote the action CLC/CLF, and an absolute requirement of the membrane form of CNTFR was required to generate a functional response to the composite cytokine. This study reinforces the functional similarity between CNTF and the CLC/CLF composite cytokine defining the second ligand for CNTFR.

Ciliary neurotrophic factor was initially named based on its ability to maintain the survival of parasympathetic neurons of chick ciliary ganglions (1, 2). Subsequent studies have revealed that CNTF[1] also enhances the survival of sensory neurons (3), motor neurons (4), and cerebellar and hyppocampal neurons (5, 6). It can also prevent lesion-induced degeneration of motor neurons and slows disease progression in mice with inherited neuromuscular deficits (7–9). The possibility that CNTF acts as a nerve-derived myotrophic factor has also been established (10, 11).

CNTF is a member of a family of structurally related cytokines that includes leukemia inhibitory factor (LIF), interleukin (IL)-6, IL-11, oncostatin M, cardiotrophin-1 (12–14), and cardiotrophin-like cytokine (CLC) (15, 16). These cytokines share one or both of the receptor signal transducing subunits gp130 or LIF receptor (LIFR) in their respective receptor complexes (17–20). The functional CNTF receptor is a ternary complex that in addition to gp130 and LIFR receptor also includes a specificity-determining binding component designed CNTF receptor (CNTFR) anchored to the membrane through a glycosylphosphatidylinositol motif (21–25). Association of CNTF to the CNTFR components subsequently leads to gp130-LIFR dimerization and activation event via the recruitment of Janus kinases (JAK1, JAK2, and TYK2) (26–30). Tyrosine-phosphorylated gp130 and LIFR in turn serve as docking proteins for signal transducing molecules such as STAT3 and SHP-2 (27, 31–35). *In vitro* studies have also shown that the gp130-LIFR pathway can stimulate the PI 3-kinase and the MAP kinase activity (36–39).

The existence of a second ligand for CNTFR was suggested by a study comparing the phenotypic consequences of disrupting CNTF *versus* CNTFR genes (40, 41). Unlike mice lacking CNTF, those lacking CNTFR die perinatally and display severe motor neuron deficits. Thus, the CNTFR subunit plays a critical role during development by serving as a receptor for a second, developmentally important ligand. Moreover, a null mutation in the human CNTF gene does not lead to neurological disease (42). CLC is a recently identified member of the CNTF/LIF family of cytokines isolated by expressed sequence tag data base screening and is also referred to as novel neurotrophin-1 or B cell-stimulating factor-3 (15, 16). We recently showed that CLC associates with the orphan soluble receptor cytokine-like factor-1 (CLF) (43, 44) to form a composite cyto-

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[1] The abbreviations used are: CNTF, ciliary neurotrophic factor; CNTFR, CNTF receptor; sCNTFR, soluble CNTFR; LIF, leukemia inhibitory factor; LIFR, LIF receptor; IL, interleukin; CLC, cardiotrophin-like cytokine; PI, phosphatidylinositol; MAP, mitogen-activated protein; CLF, cytokine-like factor-1; mAb, monoclonal antibody; FACS, fluorescence-activated cell sorter.

kine (45). CLF expression is required for CLC secretion, and the CLC/CLF heterocomplex displays functional activities exclusively on those cells and tissues co-expressing the tripartite CNTF receptor components. Thus, the CLC/CLF composite cytokine defines the long sought second ligand for CNTFR. In the present study we examined the signaling pathways recruited by the CLC/CLF composite cytokine in human cell lines of neural origin.

MATERIALS AND METHODS

Cells and Reagents—IMR-32 and SK-N-GP human neuroblastoma cell lines and the COS-7 cell line (American Type Culture Collection, Manassas, VA) were routinely grown in RPMI culture medium supplemented with 10% fetal calf serum. Human CNTF, LIF, IL-2, and the soluble form of CNTFR (sCNTFR) were purchased from R & D Systems (Minneapolis, MN). The 4G10 anti-phosphotyrosine mAb was bought from Upstate Biotechnology Inc. (Lake Placid, NY), and anti-protein C epitope mAb was from Roche Diagnostics (Meylan, France). Antibodies raised against STAT1, STAT2, STAT3, STAT4, STAT5, STAT6, SHP-2, JAK1, JAK2, JAK3, TYK2, p85 (PI 3-kinase), and polyclonal antibodies directed against the carboxyl-terminal portions of gp130 and LIFR were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Antibodies detecting phospho-ERK1/ERK2, phospho-STAT3 (Tyr^{705}) and phospho-AKT were purchased from New England Biolabs (Beverly, MA). The monoclonal antibodies directed against the human forms of LIFR (AN-E1, IgG1), gp130 (AN-HH1, IgG2a), CNTFR (AN-C2, IgG1), and CLF (AN-FC6, IgG1) generated in our laboratory (44–46) were used for FACS staining. Receptor immunoprecipitation steps were carried out using AN-G30 (anti-gp130 and IgG1) and AN-E1 (anti-LIFR and IgG1) mAbs.

COS-7 Cell Transfection and cDNAs—The COS-7 cells used in this study were intentionally chosen for their low endogenous expression levels of gp130 and LIFR (47). The cDNAs encoding LIFR, gp130, and CNTFR were cloned in pCMX expression vector. CLC and CLF cDNAs were cloned in pcDNA3. The CLC sequence was modified by introducing a c-Myc epitope (EQKLISEEDL) followed by the protein C peptide epitope (EDQVDPRLIDGK) at the COOH terminus extremity of the protein. COS-7 cells were transfected using the DEAE-dextran method as described previously (47). For each experiment, a control plate was transfected with a β-galactosidase encoding expression vector. Because the expression of large size signaling cytokine receptors on the membrane is usually weak, receptor expression experiments were pursued only when more than 80% of the cells were stained blue 48 h after transfection. For recombinant CLC/CLF synthesis, the cells were cultured for 72 h in serum-free Yssel medium (48).

Cytokine Purification—Cell culture media were concentrated using Centricon concentrators (Millipore, Bedford, MA) and loaded at 4 °C on an anti-protein C affinity column (Roche). Following extensive washing (100 mM phosphate, 1 M NaCl, 1 mM $CaCl_2$, pH 7.6), proteins were eluted from the affinity matrix by calcium ion removal (5 mM EDTA in phosphate-buffered saline) (45). Some of the preparations were further purified by a quaternary aminoethyl high pressure liquid chromatography step eluted with a NaCl gradient. Protein concentration was determined by SDS-polyacrylamide gel electrophoresis and silver staining using a bovine serum albumin protein standard.

Flow Cytometry Analysis—Cells were successively incubated for 30 min at 4 °C with the appropriate primary antibody or isotype control antibody (10 μg/ml) and a phycoerythrin-conjugated anti-mouse antibody. Fluorescence was subsequently analyzed on a FACScan flow cytometer from Becton Dickinson (Mountain View, CA).

Tyrosine Phosphorylation Analysis—After a 24-h serum starvation, the cells were stimulated for 10 min in the presence of the indicated cytokine. The cells were next lysed in 10 mM Tris-HCl, pH 7.6, 5 mM EDTA, 50 mM NaCl, 30 mM sodium pyrophosphate, 50 mM sodium fluoride, 1 mM sodium orthovanadate, proteinase inhibitors (1 μg/ml pepstatin, 2 μg/ml leupeptin, 5 μg/ml aprotinin, 1 mM phenylmethylsulfonyl fluoride), and 1% Nonidet P-40 or Brij 96 depending on the experiments (49). After pelleting insoluble material and protein standardization, the supernatants were immunoprecipitated overnight. The complexes were then isolated with beads coupled to protein A, submitted to SDS-polyacrylamide gel electrophoresis, and transferred onto an Immobilon membrane (Millipore). The membranes were subsequently incubated with the relevant primary antibody before being incubated with the appropriate secondary antibody labeled with peroxidase for 60 min. The reaction was visualized on an x-ray film using the ECL reagent (Amersham Pharmacia Biotech) according to the manufacturer's instructions. In some experiments the membranes were stripped overnight in 0.1 M glycine-HCl, pH 2.7, and neutralized in 1 M Tris-HCl, pH 7.6, before reblotting.

TABLE I
CNTF receptor expression is necessary for membrane binding of CLC/CLF

COS-7 cells were transfected with cDNAs encoding the indicated receptors or mock control. After 48 h, expression of gp130, LIFR, and CNTFR was monitored by flow cytometry analysis. Cells were incubated with CLC-c-Myc/CLF, and the membrane cytokine binding was detected with either an anti-c-Myc, an anti-CLF (AN-F-C6) mAb, or an isotype control immunoglobulin.

	Isotype control	α-CLC-c-Myc	α-CLF
Mock	–	–	–
gp130	–	–	–
LIFR	–	–	–
CNTFR	–	+++	+++
gp130/LIFR	–	–	–
gp130/CNTFR	–	+++	+++
LIFR/CNTFR	–	+++	+++
gp130/LIFR/CNTFR	–	+++	+++

Biological Assays—For proliferation assays, TF1 or derivatives of Ba/F3 cell lines were seeded in 96-well plates at a concentration of 5–10 $\times$ 10^3 cells/well in RPMI 1640 medium containing 5% fetal calf serum (46). Serial dilutions of the cytokines tested were performed in triplicate. After a 72-h incubation period, 0.5 μCi of [^{3}H]thymidine was added to each well for the last 4 h of the culture, and the incorporated radioactivity was determined by scintillation counting. For the KB transfection experiments, CNTFR cDNA was cloned in the episomal expression vector pEBS-PL as described previously (45). Transfections were carried out using the lipid reagent Fugene 6 (Roche), and 48 h later cells were serum-starved and activated for 10 min with cytokines, as indicated. ERK1 and ERK2 tyrosine phosphorylation levels were analyzed as described above.

Reporter Gene Activity—Transient transfection of SK-N-GP cells was carried out in 24-well culture plates using the Exgen® transfection reagent (Euromedex, Souffelweyersheim, France). The cells were transfected with 300 ng of reporter gene as described previously (50). 48 h after transfection, the cells were incubated with medium alone, LIF, CNTF, CLC/CLF, or IL-2 for an additional 18 h. Transfected cells were washed twice with ice-cold phosphate-buffered saline, and 100 μl of lysis buffer was added to the wells (0.1 M KH_2PO_4, pH 7.8, 0.1% Triton X-100). The extracts were then used directly to measure the luciferase activity by integrating total light emission over 10 s using a Packard Topcount luminometer (Meriden, CT). Luciferase activity was normalized based on protein concentrations.

RESULTS

Binding of CLC/CLF to the Three Components Defining the High Affinity CNTF Receptor—To assess the binding capacity of CLC/CLF to components of the CNTF receptor complex, reconstitution experiments were carried out using the COS-7 cell line. The three components defining the high affinity CNTF receptor were expressed alone, by pairs, or together. 48 h after transfection, the cells were incubated in the presence of the CLC/CLF heteromeric cytokine tagged with a c-Myc epitope at CLC carboxyl terminus. Binding to the cell surface was monitored by flow cytometry using an anti-c-Myc antibody allowing the recognition of tagged CLC bound to the cell surface (45) (Table I and Fig. 1). Membrane expression of gp130 or LIFR or their co-expression did not allow any CLC/CLF binding, indicating that their contribution to CLC/CLF binding is not essential. In contrast, CNTFR expression in COS-7 cells led to the detection of a strong specific signal, showing a preferential association of the dimeric cytokine to the latter receptor component. Similar results were also observed when CNTFR was expressed together with gp130 and LIFR. Nevertheless, the immunofluorescence peak values were usually 3-fold higher when the tripartite receptor was expressed compared with the values observed for CNTFR expressed alone. This probably reflects a higher and increased affinity of the cytokine for its complete tripartite receptor. Binding of CLC/CLF to cell sur-

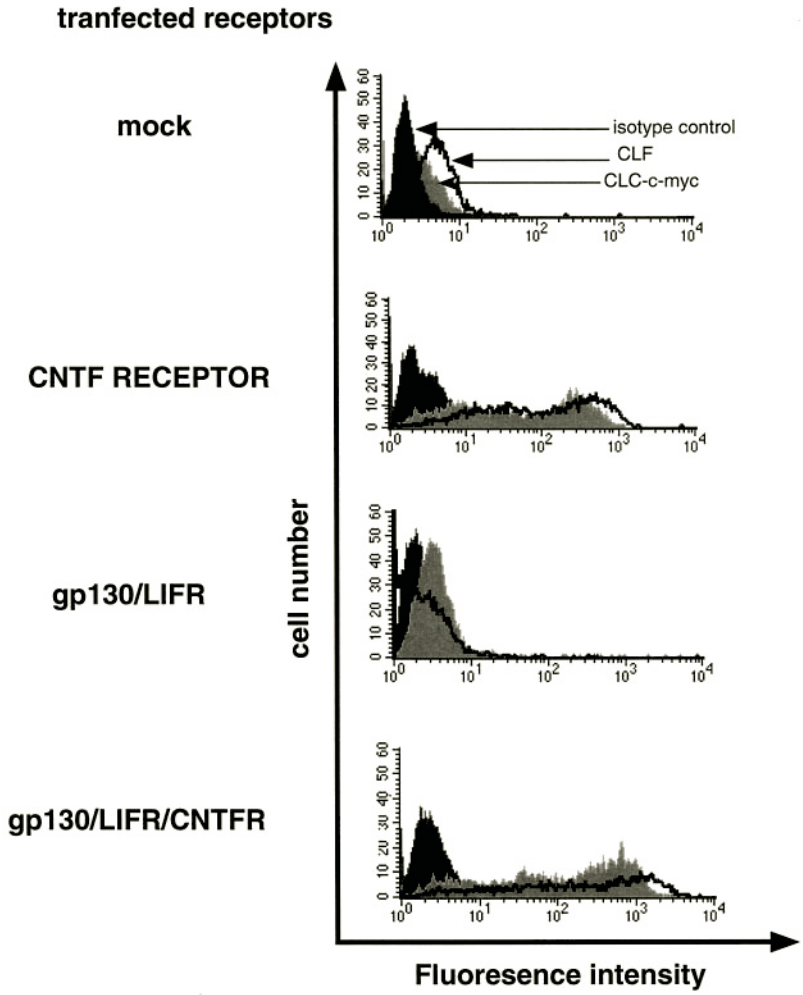

FIG. 1. **CNTF receptor expression is necessary for membrane binding of CLC/CLF.** COS-7 cells were transfected with cDNAs encoding the indicated receptors or mock control. After 48 h, the expression of gp130, LIFR, and CNTFR was monitored by flow cytometry analysis. The cells were incubated with CLC-c-Myc/CLF, and the membrane cytokine binding was detected with either an anti-c-Myc, an anti-CLF (AN-F-C6) mAbs, or an isotype control immunoglobulin.

face was also monitored with an mAb directed against CLF (Table I and Fig. 1). The results obtained were similar to those observed when using the anti-c-Myc mAb, indicating that both components remained associated when they bound to the membrane.

Recruitment of JAK Kinases by CLC/CLF—Signaling studies were then carried out using IMR-32 and SK-N-GP human neuroblastoma cell lines that express on their surface the three subunits defining the CLC/CLF functional receptor (Fig. 2). We first analyzed the ability of CLC/CLF complex to bind to the neuroblastoma cell surface. The results obtained clearly indicated membrane binding of both subunits of the composite cytokine (Fig. 2).

We next analyzed the involvement of JAK kinases in receptor activation in IMR-32 and SK-N-GP neuroblastoma cell lines (Fig. 3). A 10-min contact of cells with CLC/CLF led to an activation of JAK1, JAK2, and TYK2, as shown by analyzing the tyrosine phosphorylation content of these receptor associated kinases. Nevertheless, despite a clear TYK2 expression in neuroblastoma cells, its tyrosine phosphorylation induction was usually weaker than the signals detected for JAK1 and JAK2 in response to CLC/CLF. A similar situation was reported previously for other members of IL-6 family (49). No recruitment of JAK3 by CLC/CLF could be demonstrated (data not shown).

Once activated, JAKs are known to stimulate the phosphorylation of the gp130 and LIFR subunits of the CNTF receptor on tyrosine residues, which then serve as docking sites for SH2 containing signaling proteins (27). We reported previously that binding of CLC/CLF to its tripartite receptor complex rapidly induced tyrosine phosphorylation of both gp130 and LIFR subunits as well as their heterodimerization in the SK-N-GP cell line (45). Similarly, both gp130 and LIFR signaling components were recruited and phosphorylated by the composite cytokine in IMR-32 cells (Fig. 4).

CLC/CLF Activates STAT1 and STAT3—Downstream signaling events were further analyzed in IMR-32 and SK-N-GP neuroblastoma cell lines by studying the activation level of STAT proteins in response to CLC/CLF. As shown in Fig. 5, cell stimulation with either LIF, CNTF, or CLC/CLF elicited activation of STAT3 and to a weaker extent STAT1. Kinetic analysis of STAT3 activation shows that the observed signal peaked after a 20-min contact prior to decreasing at 2 h. This is in agreement with the typical behavior of STAT proteins following their recruitment by the IL-6 type cytokines (31, 32). Tyrosine phosphorylation analyses of the other members of the STAT family (STAT2, STAT4, STAT5, and STAT6) were carried out, but no activation could be detected in response to CLC/CLF or to CNTF, as summarized in Table II.

The transcriptional activity of STAT3 in response to CLC/CLF was then studied. For this, SK-N-GP cells were transfected with a reporter construct containing three STAT3 consensus binding sites located upstream of a thymidine kinase minimal promoter (50). 48 h post-transfection, the cells were serum-starved and stimulated for an additional 15 h with saturating amounts of LIF, CNTF, CLC/CLF, or an irrelevant cytokine (Fig. 6). A 3–4-fold increase of luciferase expression was induced by CLC/CLF, as well as by LIF and CNTF. Alto-

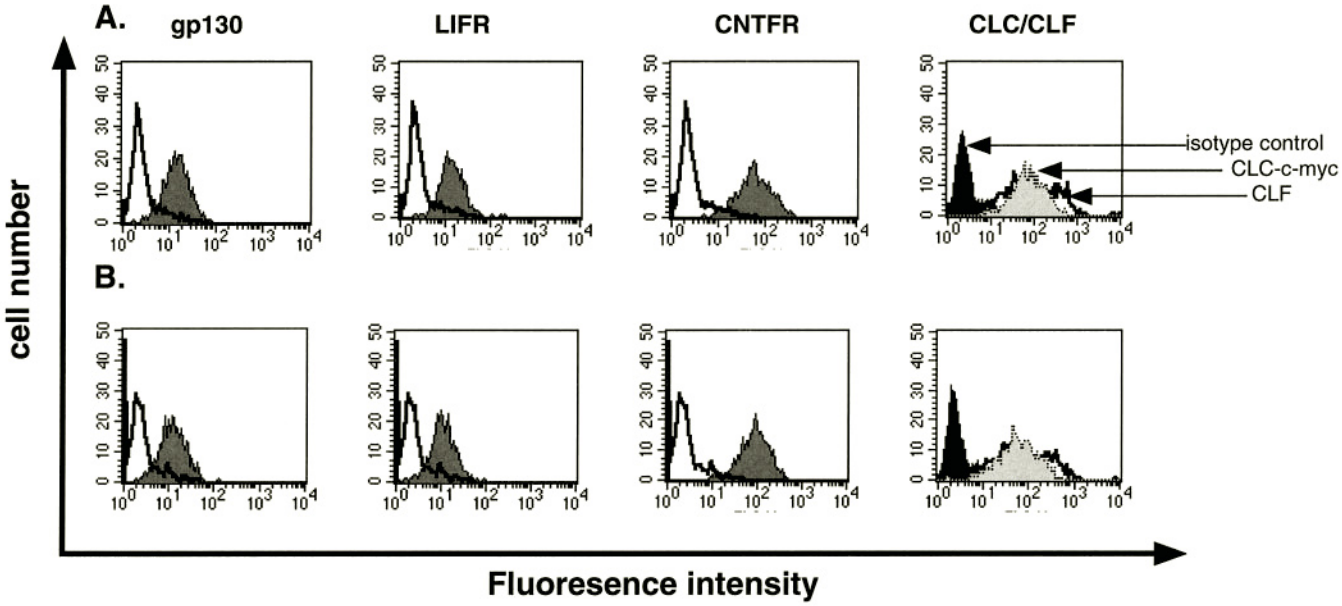

FIG. 2. **SK-N-GP and IMR-32 neuroblastoma cell lines express the tripartite CNTF receptor and bind the CLC/CLF heterocomplex.** The expression of gp130, LIFR, and CNTFR on SK-N-GP (*A*) and IMR-32 (*B*) was monitored by flow cytometry analysis. The *white histograms* correspond to the isotype controls, and the *shaded histograms* correspond to receptor detection. Binding of CLC-c-Myc/CLF was detected by incubating the cell lines with the composite cytokine, which was then detected using anti-c-Myc or anti-CLF (AN-F-C6) mAbs.

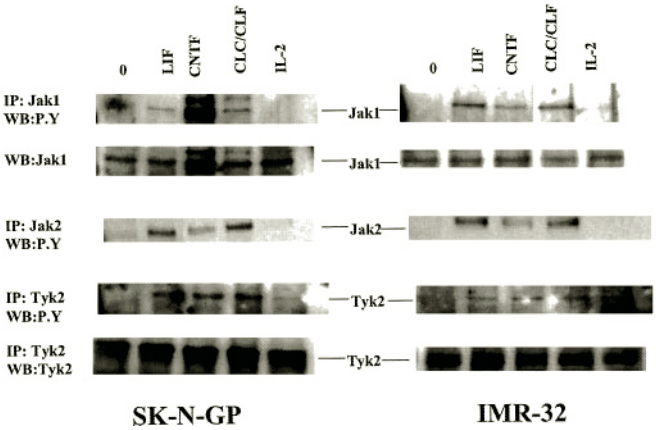

FIG. 3. **CLC/CLF induces tyrosine phosphorylation of JAK1, JAK2, and TYK2.** The SK-N-GP and IMR-32 neuroblastoma cell lines were incubated either with or without 20 ng/ml of LIF, CNTF, CLC/CLF, or IL-2 for 10 min. After lysis in 1% Nonidet P-40, proteins were immunoprecipitated as indicated, and their tyrosine phosphorylation levels were analyzed. *IP*, immunoprecipitation; *WB*, Western blot; *P.Y.*, phosphotyrosine.

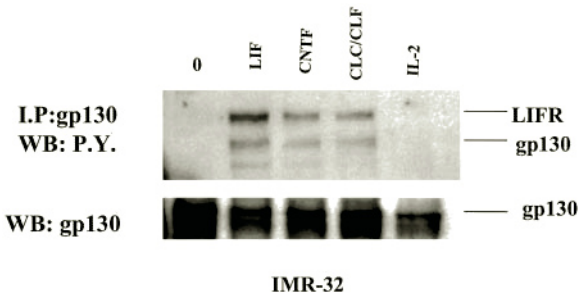

FIG. 4. **gp130 and LIFR tyrosine phosphorylation in IMR-32 cells.** Following exposure to phosphate-buffered saline or 20 ng/ml of LIF, CNTF, or CLC/CLF, IMR-32 cells were lysed, the proteins were immunoprecipitated as indicated, and their tyrosine phosphorylation levels were analyzed. *I.P*, immunoprecipitation; *WB*, Western blot; *P.Y.*, phosphotyrosine.

gether, these data indicate that CLC/CLF recruits STAT3 for both signaling and transcriptional activation of target genes.

Involvement of SHP-2 and AKT Pathway in CLC/CLF Signaling—The tyrosine phosphatase SHP-2 is known to be recruited into the tyrosine kinase signaling pathway via its binding to phosphotyrosine motifs expressed by gp130 and LIFR (27). CLC/CLF induced the tyrosine phosphorylation of SHP-2 as reported previously with CNTF and LIF (27) (Fig. 7, *A* and *B*). The association of SHP-2 with gp130 was further demonstrated by the co-immunoprecipitation of gp130 with the phosphatase (Fig. 7*C*). Similarly, an association between LIFR and SHP-2 was also seen.

It was reported previously that SHP-2 could regulate gp130 signaling by recruiting the PI 3-kinase/Akt pathway (51). We therefore analyzed the interaction between PI 3-kinase and SHP-2. The cell lysates were incubated with an anti-SHP-2 antibody, and the purified fraction was analyzed for its content of p85, the regulatory subunit of PI 3-kinase. We observed that the p85 regulatory subunit could be co-purified with SHP-2 following stimulation of neuroblastoma cells with CLC/CLF (Fig. 7*C*).

PI 3-kinase recruitment by CLC/CLF also led to a marked increase in its tyrosine phosphorylation content, as well as in its association with AKT (Fig. 8). Comparable results were obtained when treating the cells with LIF or CNTF in both tested cell lines. AKT tyrosine phosphorylation was optimal after a 10-min contact with CLC/CLF or CNTF, before to gradually decreasing after 80–100 min (Fig. 8*C*).

CLC/CLF Induces the MAP Kinase Pathway Activation—In addition to the PI 3-kinase/AKT activation pathway, SHP-2 is also known to associate with the GRAB2/Sos adaptators and regulate the MAP kinase pathway (36, 52, 53). ERK1 and ERK2 involved in the MAP kinase pathway have been shown to play important roles in mediating the mitogenic effects of the IL-6 family members. ERK1 and ERK2 activation was determined by measuring their tyrosine phosphorylation levels. Stimulation of the neuroblastoma cell lines with CLC/CLF quickly increased basal values (Fig. 9). Interestingly, the activation was quick and transient. The values went back to basal levels after a 20–30-min contact. These results demonstrate involvement of the MAP kinase signaling pathway in func-

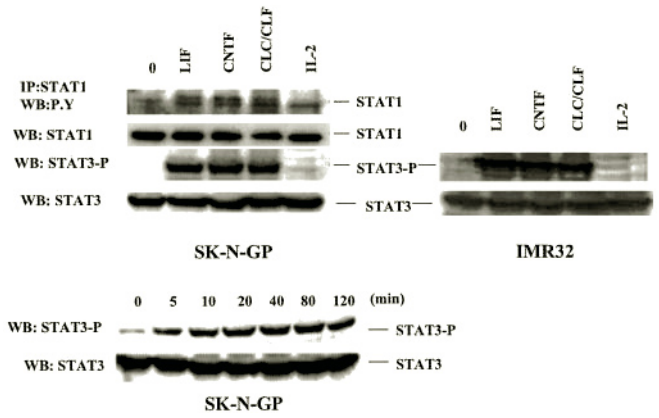

FIG. 5. **CLC/CLF induces tyrosine phosphorylation of STAT1 and STAT3 transducing proteins.** SK-N-GP cells were incubated either with or without 20 ng/ml of LIF, CNTF, CLC/CLF, and IL-2 for 10 min. After lysis in 1% Nonidet P-40, proteins were immunoprecipitated with an anti-STAT1 polyclonal antibody, and their tyrosine phosphorylation level were analyzed. SK-N-GP and IMR-32 were stimulated as described previously. After lysis in 1% Nonidet P-40, the lysates were subjected to immunoblot analysis with antibodies specific for activated forms of STAT3 (*STAT3-P*). The blots were stripped and reprobed with an antibody recognizing both activated and nonactivated STAT3 proteins. SK-N-GP cells were activated with 20 ng/ml of CLC/CLF for 5–80 min. After lysis in 1% Nonidet P-40, the lysates were subjected to immunoblot analysis as described above. *IP*, immunoprecipitation; *WB*, Western blot; *P.Y.*, phosphotyrosine.

TABLE II
CLC/CLF induces tyrosine phosphorylation of STAT1 and STAT3 transducing proteins

After activation of the IMR-32 and SK-N-MC neuroblastoma cells by the CLC/CLF composite cytokine, the recruitment of STAT signaling proteins was studied by Western blot analysis. ND, not determined.

	CLC/CLF	
	IMR-32	SK-N-GP
STAT1	ND	+
STAT2	–	–
STAT3	+++	+++
STAT4	ND	–
STAT5	–	–
STAT6	ND	–

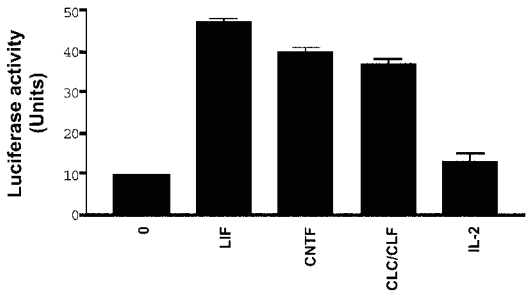

FIG. 6. **Effect of CLC/CLF stimulation on STAT3 transcriptional activity.** SK-N-GP neuroblastoma cells were transiently transfected with a reporter plasmid gene. 48 h later, the cells were treated with 20 ng/ml of LIF, CNTF, IL-2, or CLC/CLF for an additional 18 h. The cellular extracts were prepared and used to directly measure luciferase activity.

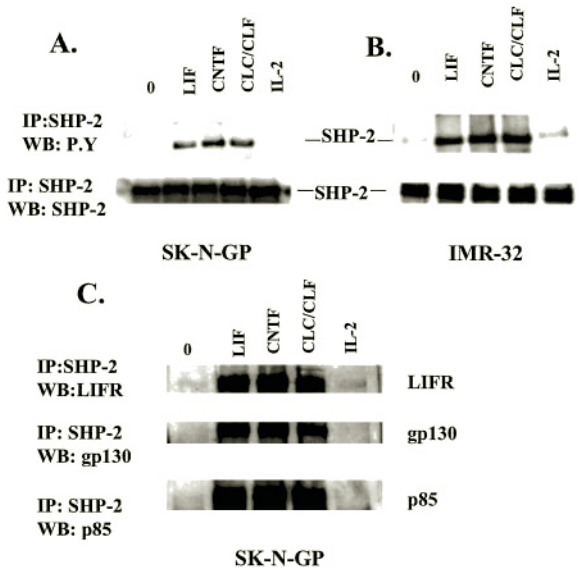

FIG. 7. **CLC/CLF induces tyrosine phosphorylation of SHP-2 transducing proteins.** The SK-N-GP (*A*) and IMR-32 (*B*) neuroblastoma cell lines were incubated either with or without 20 ng/ml of LIF, CNTF, CLC/CLF, or IL-2 for 10 min. After lysis in 1% Nonidet P-40, the proteins were immunoprecipitated as indicated, and their tyrosine phosphorylation levels were analyzed. Association of SHP-2 with others proteins in the SK-N-GP cells (*C*) was analyzed using the indicated antibodies for detection. *IP*, immunoprecipitation; *WB*, Western blot; *P.Y.*, phosphotyrosine.

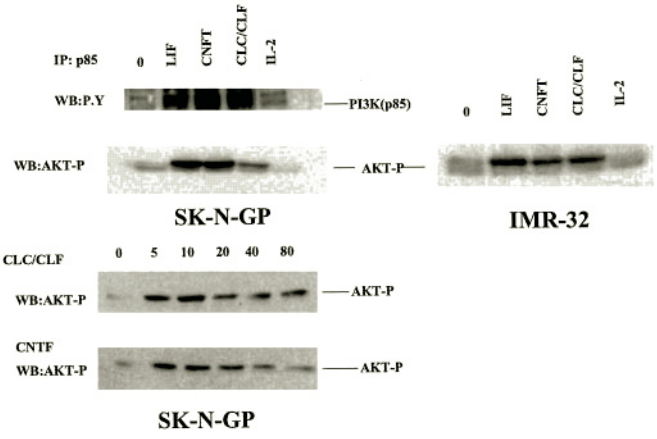

FIG. 8. **CLC/CLF stimulated phosphorylation of PI 3-kinase and AKT.** SK-N-GP cells were incubated either with or without 20 ng/ml of LIF, CNTF, CLC/CLF, and IL-2 for 10 min. After lysis in 1% Nonidet P-40, proteins were immunoprecipitated with an anti-P85 polyclonal antibody, and their tyrosine phosphorylation levels were analyzed. SK-N-GP and IMR-32 cells were stimulated as described above. After lysis in 1% Nonidet P-40, lysates were subjected to immunoblot analysis with antibodies specific for activated AKT (*AKT-P*). SK-N-GP cells were activated with 20 ng/ml of CLC/CLF or CNTF for 5–80 min. After lysis in 1% Nonidet P-40, the lysates were subjected to immunoblot analysis with antibodies specific for activated AKT. *IP*, immunoprecipitation; *WB*, Western blot; *P.Y.*, phosphotyrosine.

tional responses to the CLC/CLF composite cytokine.

Soluble CNTFR Failed to Promote the CLC/CLF Response—In a collaborative study we reported previously that cell lines not normally responsive to CNTF responded to treatment with a combination of CNTF and the sCNTFR component (23). Additionally, we also observed that CLC/CLF could, *in vitro* or on the cell surface, directly contact sCNTFR (Ref. 45 and Fig. 1). Therefore, experiments were carried out using the TF1 erythroleukemia cell line (23), and derivatives of the IL-3-dependent Ba/F3 cell line were rendered responsive to LIF or to CNTF by transfection with the appropriate receptor chains (Ref. 46 and Fig. 10). Both TF1 and BA/F3 cell lines expressing gp130 and LIFR responded to LIF and to a combination of CNTF and sCNTFR, as reported before (Ref. 23 and Fig. 10, *A* and *B*). Interestingly, sCNTFR entirely failed to promote any response to CLC/CLF in these two cell lines. In contrast, CLC/CLF was fully active on a BA/F3 cell line expressing the tripartite CNTF receptor complex on its surface (Fig. 10*C*). These results indicate that CLC/CLF cannot be substituted by CNTF. In spite of an *in vitro* recognition between CLC/CLF and

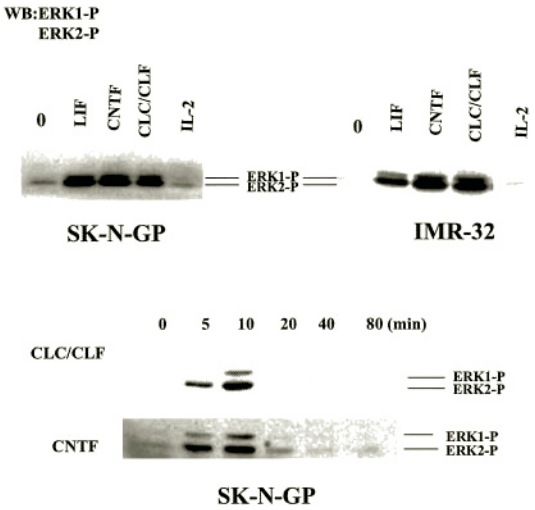

FIG. 9. **CLC/CLF induces the phosphorylation of ERK1 and ERK2.** SK-N-GP and IMR-32 cells were incubated either with or without 20 ng/ml of LIF, CNTF, CLC/CLF, and IL-2 for 10 min. After lysis in 1% Nonidet P-40, the lysates were subjected to immunoblot analysis with antibodies specific for activated ERK1 and ERK2. SK-N-GP cells were activated with 20 ng/ml of CLC/CLF or IL-2 for 5–80 min. After lysis in 1% Nonidet P-40, the lysates were subjected to immunoblot analysis with antibodies specific for activated ERK1 and ERK2 (ERK1-P and ERK2-P). *WB*, Western blot.

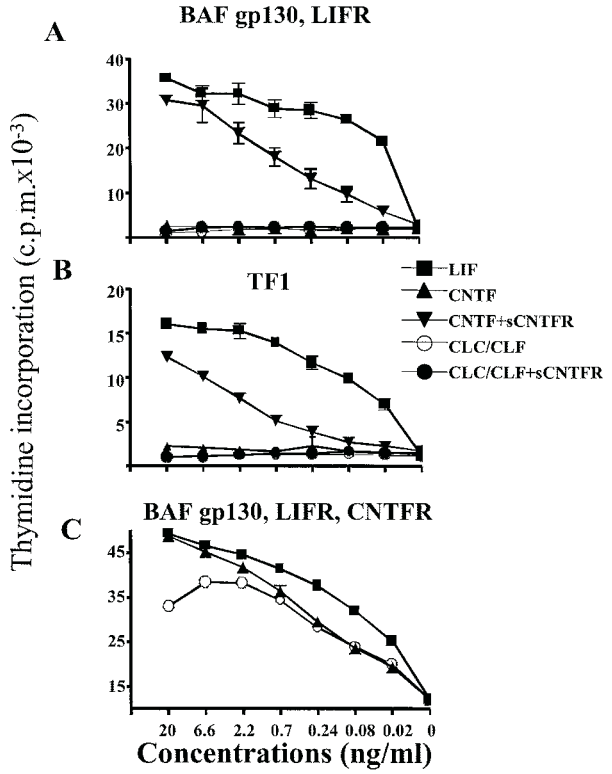

FIG. 10. **Membrane CNTFR expression is required for the proliferative response of hematopoietic cell lines to CLC/CLF.** TF1 cells and Ba/F3 cells expressing gp130, LIFR, or the tripartite CNTF receptor (gp130, LIFR, and CNTFR) were seeded in 96-well plates at a concentration of 5 or 10 $\times$ 10^3 cells/well. Serial dilutions of tested cytokines were added in triplicate. After a 72-h incubation period, 0,5 μCi of [^{3}H]thymidine, was added and 4 h later the incorporated radioactivity was determined by scintillation counting.

sCNTFR (45), the composite cytokine plus sCNTFR was not able to elicit a functional proliferative response in cell lines expressing only gp130 and LIFR on their surfaces.

Additional experiments were performed based on the ability

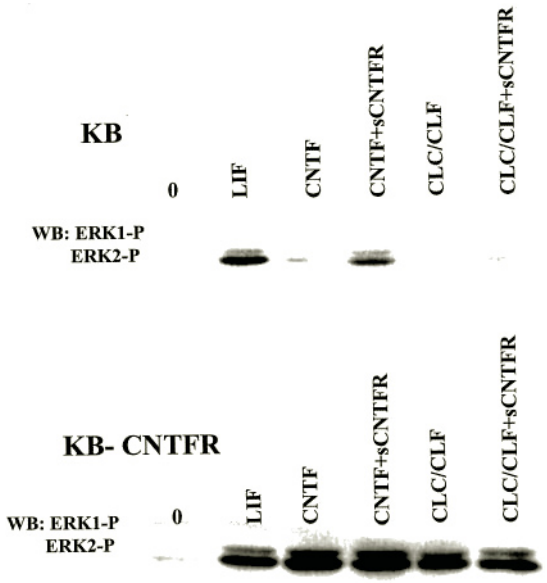

FIG. 11. **Membrane CNTFR expression is required to induce the MAP kinase pathway activation in response to CLC/CLF.** KB epidermoid carcinoma cells or CNTFR transfected KB cells were stimulated for 10 min in the presence of 20 ng/ml cytokine and 500 ng/ml sCNTFR, as indicated. The cells were lysed, and their ERK1 and ERK2 tyrosine phosphorylation levels were determined. *WB*, Western blot.

of the KB epidermoid carcinoma cell line to become activated when grown in the presence of LIF (45). MAP kinase phosphorylation levels were analyzed in the KB cells (Fig. 11). As previously shown, KB cells become sensitive to CNTF or CLC/CLF only when transfected with membrane-bound CNTFR (45). Importantly, association of sCNTFR to CNTF also induced MAP kinase pathway activation. In contrast, no activation could be detected when sCNTFR was used in combination with CLC/CLF. These results clearly established an absolute requirement of the membrane-bound form of CNTFR to generate a functional response to CLC/CLF composite cytokine.

DISCUSSION

We reported previously that CNTF and the CLC/CLF heterocomplex act through the same receptor complex to generate overlapping biological functions (45). In the present study we have analyzed the cell signaling mechanisms observed in response to the CLC/CLF composite cytokine and compared the recruited pathways with those activated by both CNTF and LIF. Our analysis demonstrates a large overlap between the pathways activated by LIF, CNTF, and CLC/CLF.

As reported previously for CNTF, the CLC/CLF composite cytokine preferentially binds to the CNTFR subunit (22, 25, 54). In contrast, LIFR, known as a binding component for both LIF and cardiotrophin-1, failed to recognize the dimeric cytokine (49). Similarly, gp130, which can bind oncostatin M, was not able to contact CLC/CLF directly (19). The contribution of both gp130 and LIFR to CNTF binding is essential to increase the affinity of the ligand to the membrane (25, 54). A similar process might occur for CLC/CLF, because a co-expression of gp130 and LIFR with CNTFR leads to a 3–4-fold increase of the mean fluorescence value recorded by FACS analysis. The use of radiolabeled ligand should help to determine the affinity constants of CLC/CLF toward the three components of the functional receptor. By using gp130 neutralizing antibodies and analyzing the tyrosine phosphorylation content of gp130 and LIFR in response to CLC/CLF, we have shown that the formation of a gp130-LIFR heterocomplex is essential for the composite cytokine signaling (45).

In neuroblastoma cells expressing the tripartite CNTF re-

ceptor components on their surface, the CLC/CLF composite cytokine triggered gp130-LIFR association and tyrosine phosphorylation induced by JAK tyrosine kinases. As reported previously for related ligands, an activation of JAK1, JAK2, and TYK2 was observed in response to CLC/CLF (26–30). According to the respective phenotypes demonstrated in JAK kinase-deficient mice (56–58), we can hypothesize a prevalent involvement of JAK1 in gp130-LIFR signaling in response to CNTF and CLC/CLF (55). Additionally, in Jak1 $^{-/-}$ mice, neurons are unable to respond to the ligands of the gp130 receptor family and die by apoptosis, whereas neuronal development was not affected in mice lacking JAK2, JAK3, or TYK2 (56–58).

Following CLC/CLF cell activation and JAK activation, information is relayed to the nucleus by a number of signaling molecules, including the STAT3 transcriptional activator, which is quickly recruited by the second CNTFR ligand. STAT3 is essential for the early development of the mouse embryo (59). The embryos developed until embryonic day 6 before a rapid degeneration with no obvious mesoderm formation. Involvement of STAT3 in CNTF signaling was demonstrated initially using neuroblastoma cell lines or receptor reconstituted systems expressed in fibroblast cell lines (27, 31). Their functional responses to CNTF have since been detected in cells of glial origin (60, 61). The present work led to similar conclusions by demonstrating that STAT3 is also a major signaling protein for the second CNTF receptor ligand. The effect of the CLC/CLF composite cytokine on glial cells remains to be established. We also detect to a lesser level a STAT1 activation in CLC/CLF signal transduction. STAT1 gene inactivation in the mouse has underlined its essential involvement in mediating the antiviral properties of interferons, although no evidence was found for an alteration of the responses mediated through the gp130 pathway in these mice (62).

The tyrosine phosphatase SHP-2 is widely expressed and becomes tyrosine-phosphorylated after cell stimulation with cytokines (27). There is some controversy about the role of SHP-2 in tyrosine kinase signaling and the relative contributions of its SH2 domains, catalytic domain, and carboxyl-terminal tail to its downstream effects (63). Thus, SHP-2 may be a positive effector of signal transduction by acting as an adaptator protein to associate with GRAB2, leading to activation of the MAP kinase cascade (53, 54). The recruitment of SHP-2 by the gp130 pathway leads to the transmission of proliferative signals in various hematopoietic cell systems (64). The use of either a modified gp130 receptor lacking the SHP-2 binding site or of SHP-2 dominant negative mutants has demonstrated a negative regulatory role for SHP-2 (65, 66). The dual function of SHP-2 was further reinforced by studies involving Shp-2 defective mouse embryos, whereby SHP-2 can function as either a positive or a negative regulator for the MAP kinase activation, depending on the specific receptor pathway stimulated (63). In the present work, the CLC/CLF composite cytokine strongly recruited SHP-2. Co-precipitation experiments show an association between gp130 and the tyrosine phosphatase most probably through tyrosine 759 of gp130 as reported before (27). Interestingly, tyrosine 759 has also been shown to bind the suppressor of cytokine signaling-3 (67, 68). A number of studies reporting a negative regulatory function of SHP-2 were based on observed increases in gp130-dependent signaling when Tyr^{579} is mutated (65, 66). Part of the observation might therefore be linked to the negative regulatory function of the suppressor of cytokine signaling-3. A recent study reports that LIFR can directly bind to SHP-2 (69). Similarly, an association between SHP-2 and LIFR was demonstrated following activation of the tripartite receptor complex by CLC/CLF. It remains to be determined whether SHP-2 transduces a positive or a negative regulatory signal after its recruitment by the CLC/CLF composite cytokine. A robust recruitment of the PI 3-kinase/AKT pathway was also observed in response to CNTF and to the second CNTFR ligand. A contact between p85 PI 3-kinase and SHP-2 was also seen as reported previously for the IL-6 receptor or the thrombopoietin receptor (39, 70). AKT recruitment was time-dependent and remains elevated after a 1-h contact period with the composite cytokine. PI 3-kinase has previously been reported to act upstream of MAP kinase activation when the gp130/LIFR complex bound LIF (39). In the present study we observed a rapid induction of ERK1 and ERK2 tyrosine phosphorylation following cellular activation by CLC/CLF. This activation process was transient and entirely disappeared after 20 min of contact, pointing toward the existence of a negative regulatory loop. This result corroborates the observation that the AKT pathway was able to down-modulate the Raf pathway (71). Cross-talk between the MAP kinase and the PI 3-kinase pathways may switch the biological response between proliferation and differentiation processes.

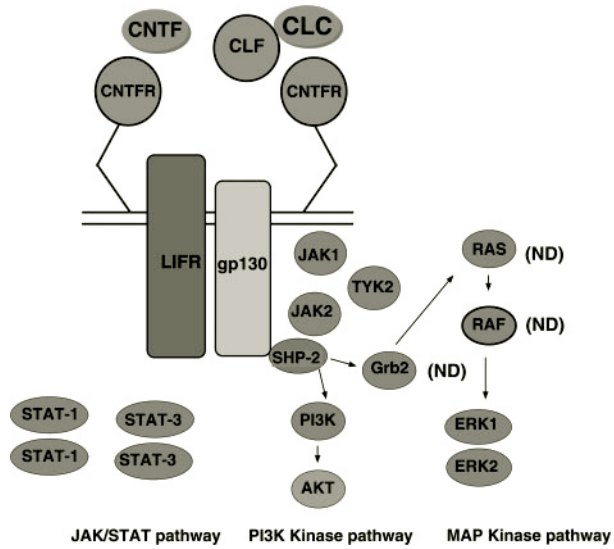

FIG. 12. **Schematic presentation of signaling pathways recruitment by CLC/CLF.** After binding of CLC/CLF or CNTF to the tripartite CNTF receptor (gp130, LIFR, and CNTFR), the JAK/STAT, the PI 3-kinase, and the MAP kinase pathways were recruited.

In a collaborative study we have previously shown that sCNTFR was able to promote the CNTF response in cell lines that only expressed LIFR and gp130 on their surface (23). By performing co-immunoprecipitation experiments we reported the possibility that the CLC/CLF composite cytokine associates with sCNTFR (45). In the present work we clearly established that association of CLC/CLF to sCNTFR is not sufficient to elicit responses in gp130^{+} LIFR^{+} cell lines and that an absolute requirement of a membrane form of CNTFR was necessary to induce a functional response to the CLC/CLF composite cytokine. This represents a major difference between the activation processes developed by CNTF and CLC/CLF. This also suggests that, in response to CLC/CLF, the membrane form of CNTFR would be able by itself to signal through its glycosylphosphatidylinositol anchorage. Interestingly, similar observations have been made for a number of glycosylphosphatidylinositol-linked proteins (72).

In conclusion, we show in neuroblastoma cell lines, both CNTF and CLC/CLF composite cytokine are able to recruit the STAT3, PI 3-kinase, and MAP kinase pathways (Fig. 12). This study reinforces the functional similarity between CNTF and the composite cytokine defining the long sought CNTF-2 (40, 45). The major difference between these two cytokines resides

in the fact that, in contrast to CNTF, CLC/CLF requires an absolute requirement of a membrane-bound form of CNTFR to elicit a functional response. A second difference is the ability of CLC/CLF to get secreted outside of the cells, whereas CNTF is mainly released from cells during trauma (73). In contrast to CNTF, CNTFR is highly expressed in the developing embryo (74), and we are therefore investigating embryonic expression of both CLC and CLF. Preliminary observations indicate that both components are expressed during embryonic life. Finally, it will be important to positively identify CLC/CLF as CNTF-2 and to exclude the possibility of yet more ligands for the CNTF receptor by demonstrating that a double knock out for CNTF and CLC encompasses the phenotype resulting from CNTFR gene disruption.

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