Biochemical and Biophysical Research Communications **262,** 132–138 (1999)
Article ID bbrc.1999.1181, available online at http://www.idealibrary.com on IDEAL®

Computational EST Database Analysis Identifies a Novel Member of the Neuropoietic Cytokine Family

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Received July 16, 1999

A novel member of the neuropoietic cytokine family has been cloned and the protein expressed and characterized. In an effort to identify novel secreted proteins, an algorithm incorporating neural network algorithms was applied to a large EST database. A full-length clone was identified that is 1710 bp in length and has a single open reading frame of 225 amino acids. This new cytokine is most homologous to cardiotrophin-1, having a similarity and an identity of 46 and 29%, respectively, and therefore we have named it cardiotrophin-like cytokine (CLC). Northern hybridization analysis identified a 1.4-kb messenger RNA that is highly expressed in spleen and peripheral leukocytes. Purified recombinant CLC induced the activation of NFκB and SRE reporter constructs in the TF-1, U937, and M1 cell lines. Furthermore, the signal transduction pathway for CLC was characterized in the neuroblastoma cell line SK-N-MC and found to involve tyrosine phosphorylation of gp130 and STAT-1.

The neuropoietic cytokine family is currently composed of 6 factors; IL-6, IL-11, oncostatin M (OSM), ciliary neurotrophic factor (CNTF), leukemia inhibitory factor (LIF), and cardiotrophin-1 (CT-1). Members of this family have limited sequence homologies and are predicted to share a four antiparallel α helix bundle secondary structure (1, 2). With the exception of IL-11 and CNTF, the other neuropoietic cytokine genes have presumptive N-glycosylation sites and, excluding CNTF and CT-1, have putative signal sequences predictive of secreted proteins (3, 4).

Neuropoietic cytokines induce cellular responses through the activation of homo- (IL-6 and IL-11) or hetero- (CT-1, OSM, CNTF, LIF) dimeric receptor complexes that include a ligand selective α component (e.g., CNTFRα) and LIFR as a B component (4–10). Both the homo- and hetero-receptor complexes bind gp130 which then dimerize and induce the activation of receptor-associated tyrosine kinases of the JAK family (for review see 11). Other cytoplasmic proteins (e.g., PLCγ and STAT) are then recruited into the receptor complex and subsequently tyrosine phosphorylated (7). The selection and activation of a particular member of the STAT family is regulated by receptor substrate-specifying motifs (10). Once phosphorylated, they dimerize and translocate into the nucleus and bind to specific binding sequences located in the transcriptional control regions of various target genes (12–14).

Cytokines in this family have distinct yet overlapping actions on several biological systems. LIF administration results in elevated numbers of splenic megakarocytes, splenomegaly, and excess and ectopic bone formation (15–17). Treatment with LIF or IL-11 induces an increase in the number of circulating platelets (16–19). All 6 factors induce the acute-phase serum amyloid A and potentiates the increase in serum corticosteroid levels induced by suboptimal doses of IL-1 in mice (20). IL-6 and IL-11 modulate immune responses through the regulation of immunoglobulin secretion (21, 22). LIF, IL-6, OSM, CT-1, and CNTF effect the survival and/or phenotypic differentiation of numerous neuronal populations (23–31).

As a result of a screening effort to identify novel secreted proteins in our database, we have identified, molecularly cloned and characterized the biological activity of a novel member of the neuropoietic cytokine family. This novel member, CLC, has a distinct yet overlapping expression pattern with other members of the family and shares some of the known biological activities associated with the other neuropoietic cytokines.

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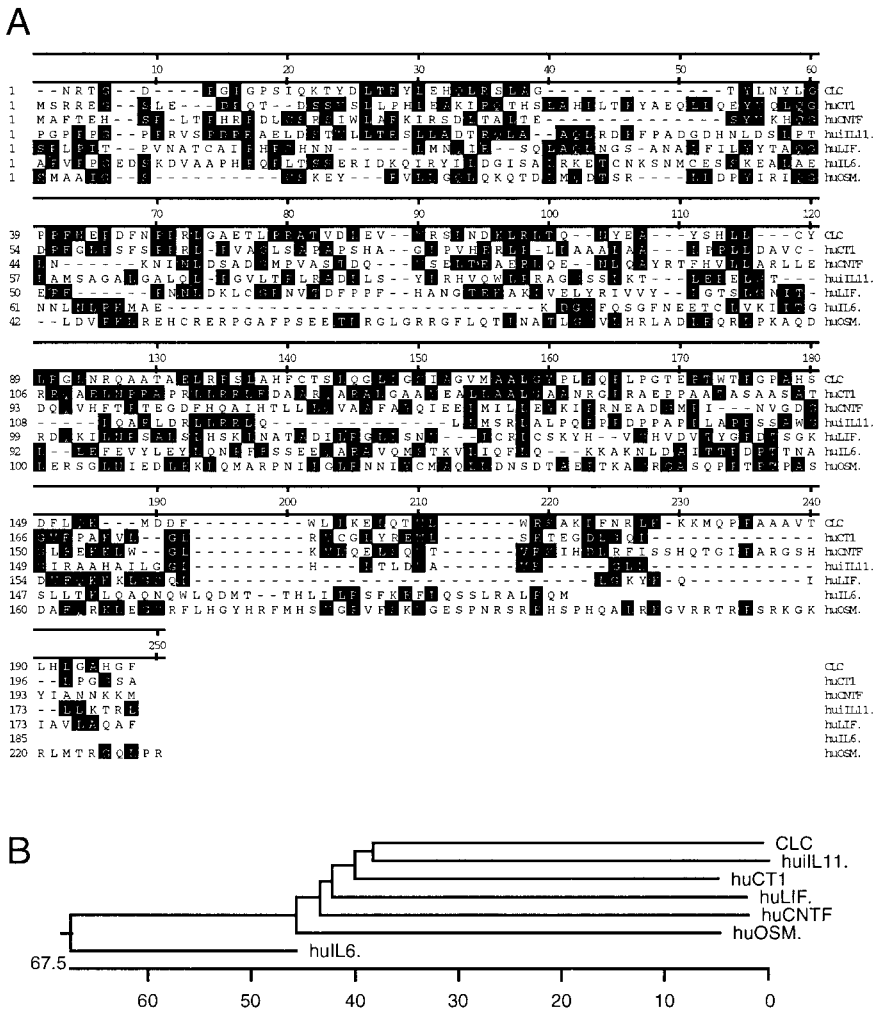

FIG. 1. Amino acid sequence of human CLC. (A) The coding and predicted mature amino acid sequence of human CLC. The predicted signal sequence is underlined. (B) Hydrophilicity plot for human CLC as determined by Signal P analysis.

MATERIALS AND METHODS

Clone isolation and sequencing. The HGS cDNA database was searched using HMM and Signal P algorithms to identify EST's with putative signal peptides. Full-length sequencing of the putative candidates using ABI 377 sequencers (PE Biosystems, Redwood, CA) led to the identification of a single clone showing significant homology to cardiotrophin-1.

Northern hybridization analysis. The cDNA insert from pHNFIR05 was radioactively labeled using RediPrime according to the manufacturer's instructions (Amersham Life Sciences, Arlington Heights, IL). The unincorporated nucleotides were removed from the labeled probe by using G25 Sephadex spin columns (Pharmacia Biotech, Piscataway, NJ). Human 12-Lane Multiple Tissue Northern blots containing poly-A^+ RNA from a variety of tissues were purchased from Clontech (Palo Alto, CA) and the hybridization was conducted according to the manufacturer's instructions.

Protein purification. The bacterial expression plasmid pQE9: CLC.L28-F225 was constructed by PCR amplification of the CLC cloning insert from the pHNFIR05 using two synthetic oligonucleotides, ACTGGGGATCCCTCAA-TCGCACAGGGGACCCAGGGC and GGGGGGAAGCTTTCAGAAGCCATG-AGCCCCCAGGTGC. Following purification by G25 Sephadex column chromatography, the PCR product was excised with *Bam*HI and *Hin*dIII, and then cloned into the pQE9 vector. The expression construct encodes for the mature form of CLC, since the initial 27 amino-terminal residues have been removed.

The recombinant CLC was expressed in M15[REP4] host (Qiagen, Valencia, CA). Following a 4 hr induction with IPTG (1 mM), cell lysates were prepared under denaturing conditions (6 M guanidine–HCl, 0.1 M NaH_2PO_4, 0.01 M Tris–HCl, pH 8.0). The protein was purified using Ni–NTA agarose affinity chromatography and an FPLC-sizing column. The purified protein was then dialyzed into a 10 mM sodium acetate buffer (pH 4.7) containing 50 mM sodium chloride.

Immunoprecipitation of tyrosine phosphorylated proteins. The preparation of cell lysates, the immunoprecipitations, and the western blotting were conducted essentially as previously described (32). The concentrations of the antibodies used are as follows; antiphosphotyrosine (0.12 μg/ml, UBI), anti-gp130 (8 μg/ml, UBI), anti-STAT-1 (5 μg/ml, UBI) or anti-STAT-3 (4 μg/ml, UBI).

Myeloid reporter cell lines. TF-1, M-1, U-973 cell lines were obtained from American Type Culture Collection (Manassas, VA) and maintained in culture as per their instructions. The cells were co-transfected with plasmid DNA for pSRE-SEAPII, pNFkβ-SEAPII or pGAS-SEAPII, and a selection marker plasmid pcDNA3.1 at a molar

A

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        10                  30                  50
MDLRAGDSWG MLACLCTVLW HLPAVPALNR TGDPGPGPSI QKTYDLTRYL EHQLRSLAGT

        70                  90                 110
YLNYLGPPFN EPDFNPPRLG AETLPRATVD LEVWRSLNDK LRLTQNYEAY SHLLCYLRGL

       130                 150                 170
NRQAATAELR RSLAHFCTSL QGLLGSIAGV MAALGYPLPQ PLPGTEPTWT PGPAHSDFLQ

       190                 210
KMDDFWLLKE LQTWLWRSAK DFNRLKKKMQ PPAAAVTLHL GAHGF
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B

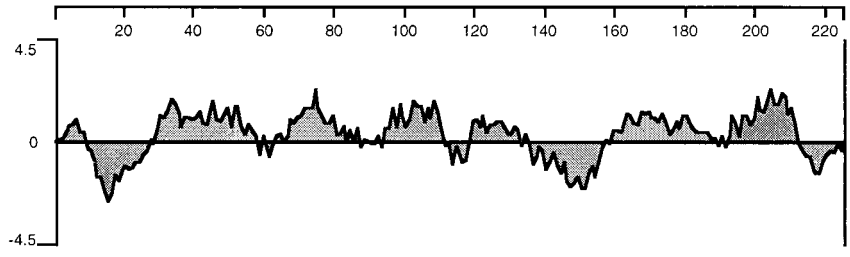

FIG. 2. Multiple sequence alignment and phylogenetic tree comparing CLC with other members of the neuropoietic cytokine family. (A) Pairwise comparison of the amino acid sequence of CLC to other neuropoietic cytokines. The shaded areas denote residues that are in common between the various family members and CLC. (B) Depicts the evolutionary relationship between CLC and the other neuropoietic cytokines.

ratio of 5:1. The transfected cells were selected with 500 μg/ml neomycin and the resistant cells were cloned. The cloned reporter cell lines were maintained under selection in culture medium containing 300 μg/ml neomycin.

Reporter assays. The various reporter cell lines were seeded at a density of 7.0 (10^3) cells/mm^2 in 200 μl of culture medium. The cells were maintained in serum-free medium for 18–24 h prior to the assay. The factors were then added and the final volume in the wells was adjusted to 250 μl. After 48 h, an aliquot of the conditioned medium was taken to determine the level of secreted alkaline phosphatase enzyme activity according to the manufacturer's specifications (Boehringer-Mannheim, Indianapolis, IN).

Chromosomal mapping. The genomic position of the CLC gene was determined by using the standard G3 radiation hybrid panel (Research Genetics, Huntsville, AL). The panel DNAs were amplified by PCR using CLC gene specific primers 5′-GAACTACCTGGGCCCCCCTTTC-3′ and 5′-CATCACACGCCCAGCCGGT-CCAGG-3′.

RESULTS

Using the Signal P and HMM algorithms (33, 34) designed to identify the hallmark features of signal sequences, the HGS EST database was searched and over 12,000 putative sequences were identified. Full-length sequencing of these clones and analysis of the sequence information using TBLASTN (35) identified a full-length cDNA encoding a homolog of CT-1, HNFIR05. The clone was 1710 bp in length and contained a single open reading frame of 225 amino acids (Fig. 1A). Signal P algorithm analysis indicated that residues (1–27) at the amino-terminus demonstrated a substantial degree of hydrophobicity and thus may constitute a secretory signal peptide (Fig. 1B). Furthermore, the analysis identified a second region of hydrophobicity in the region of amino acids 135–160.

A pairwise comparison between the deduced amino acid sequence for CLC and the other members of the neuropoietic cytokine family demonstrate a 46% similarity and a 29% identity to hCT-1 and a 20–26% identity to the other family members. The areas of homology did not appear to be clustered in any particular region of the protein but rather showed a relatively uniform distribution as depicted in Fig. 2A. The phylogenetic tree shown in Fig. 2B graphically depicts the ancestral relationships among the various members of the neuropoietic cytokine family. The length of each pair of branches represents the distance between sequence pairs measured by the number of nonconservative amino acid substitution events. In this phenogram, the distances between ancestors in the tree are averaged. This comparison demonstrates that CLC is

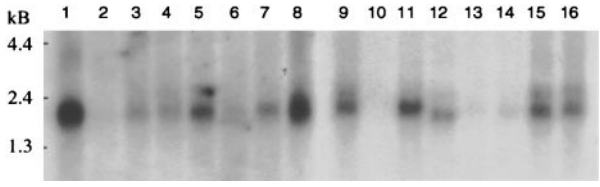

FIG. 3. Tissue distribution of CLC transcripts based on Northern hybridization. The Northern hybridization analysis was conducted using the full-length cDNA as a probe and filters containing 2 μg/lane of poly-A$^+$ RNA from human adult tissues. The tissue loading sequence is as follows: lane 1, spleen; 2, thymus; 3, prostate; 4, testis; 5, ovary; 6, small intestine; 7, colon; 8, peripheral blood leukocytes; 9, heart; 10, brain; 11, placenta; 12, lung; 13, liver; 14, smooth muscle; 15, kidney; 16, pancreas.

most closely related to human IL-11 with less than 40 nonconservative amino acid substitutions.

Using Northern hybridization analysis, we characterized the distribution of CLC in numerous adult human tissues. The highest level of expression of the 1.9 kb transcript was in the spleen and peripheral blood leukocytes, while moderate levels were detected in ovary, placenta and kidney (Fig. 3). Low message levels were observed in the colon, heart, lung and pancreas. Interestingly, the transcript size for CLC in the lung appears to be smaller than that observed in the other tissues suggesting that further processing of the messenger RNA may be occurring in this tissue. Furthermore, CLC clones were also detected in cDNA libraries prepared from activated or resting neutrophils, bone marrow stromal cells, and synovial fibroblasts (data not shown).

To determine the chromosomal localization of human CLC, we used a standard G3 radiation hybrid panel. The CLC gene has the linkage to SHGC-14407, SHGC-11734, and SHGC-57354 markers on chromosome 11 with LOD scores of 9.41, 8.53, and 8.45 at distances of 13, 14, and 18 centiRad, respectively. This corresponds to a cytogenetic location of 11q13.3.

The cDNA encoding the mature form of CLC (residue 28–225) was subcloned into QE9 vector, expressed in *E. coli* and the histidine-tagged form of the protein was purified as described in the Materials and Methods section (Fig. 4A). The biological activity of the purified recombinant CLC was tested on NFKβ- and SRE-TF1 reporter cell lines. CLC induced a concentration dependent increase in the amount of SEAP released into the culture medium (Figs. 4B and 4C, respectively). Maximal levels of cellular stimulation, increases of 10 to 12-fold compared to the vehicle control, were achieved with 50 ng/ml of CLC. The EC_{50} values were calculated as approximately 10 ng/ml in both reporter cell lines. In contrast, when the cultures were treated with heat inactivated (30 min at 90°C) CLC, only basal levels of SEAP release were observed. GM-CSF (2 ng/ml) was used as a positive control for these experiments and induced an increase in SEAP release between 24- and 31-fold. The results using M1 and U937 reporter cell lines under identical experimental conditions were similar to those described above for the TF-1 cells (data not shown).

Following receptor binding and activation, other members of the neuropoietic cytokine family are known to induce the tyrosine phosphorylation of gp130 and the activation of the Jak-STAT pathway. We investigated if CLC treatment activates a similar pathway in SK-N-MC cells. Figure 5A depicts the time course of the CLC and CT-1 induced increase in the tyrosine phosphorylation of gp130. Maximal levels of phosphorylation were achieved within 10 min of treatment with either CLC or CT-1. The response to either factor appeared to be somewhat diminished at the 20- and 40-min time points. Since the kinetics of the activation of gp130 by CT-1 appeared more rapid in comparison to the response induced by CLC, we investigated if the commercial preparation of CT-1 was more potent than the current preparations of CLC. The results depicted in Fig. 5B demonstrate that CT-1 at a concentration of 0.1 ng/ml induces a detectable increase in gp130 phosphorylation. Furthermore, 1 ng/ml of CT-1 and 10 ng/ml of CLC appeared to induce an equivalent level of gp130 phosphorylation. Thus the

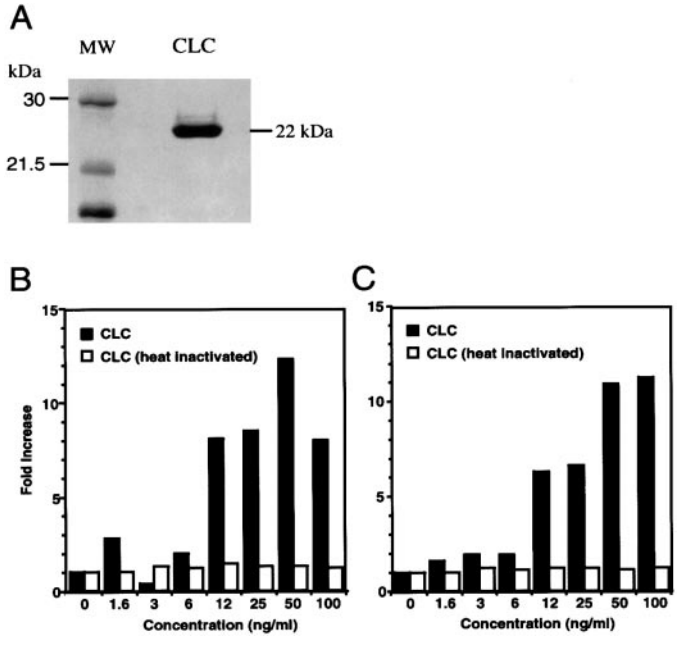

FIG. 4. Purification of human recombinant CLC and biological activity on TF-1 reporter cell lines. (A) The predicted mature form of the protein (22 kDa) was purified and subjected to SDS–PAGE chromatography on 14% gels, with 2 μg loaded per lane. (B) The NFκB-TF-1 cells were incubated in serum-free medium in the presence of the indicated concentrations of CLC for 48 h. The conditioned medium was then collected for the SEAP assay. The data points represent the ratio of the level of SEAP activity measured in the medium from the TF-1 cells treated with factors versus the buffer vehicle which were adjusted to 1. (C) The response of the SRE-TF-1 cell line to CLC was determined as described above for the NFκB cell line.

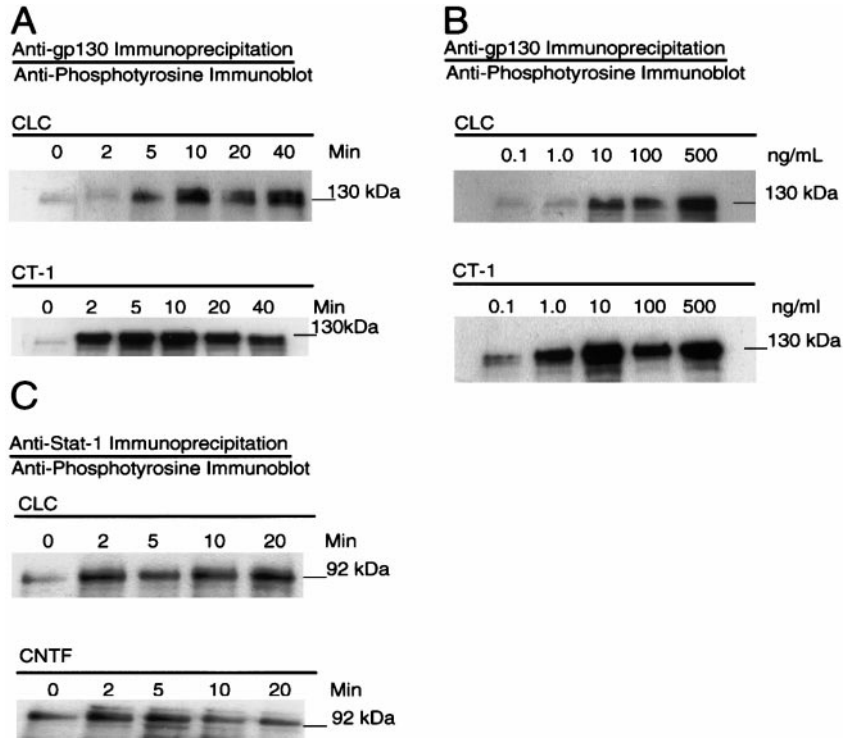

FIG. 5. CLC induces the tyrosine phosphorylation of gp130 and STAT-1. (A) Monolayer cultures of SK-N-MC cells were incubated in serum-free medium for 24 h prior to treatment with CLC (200 ng/ml) or CT-1 (100 ng/ml). Following the indicated time intervals, gp130 was immunoprecipitated, Western blotted, and then immunodetected with an antiphosphotyrosine antibody. (B) The dose dependency of the gp-130 response to CLC or CT-1 was examined following a 10-min treatment. (C) The kinetics of the CLC and CNTF induced phosphorylation of STAT-1. These experiments were conducted essentially as in part A with the exception that STAT-1 was immunoprecipitated.

difference in the potency of the two ligands could explain the apparent difference observed in the kinetics of the gp130 phosphorylation.

Downstream signaling events were also investigated by testing if CLC induces the tyrosine phosphorylation of STAT 1 and STAT 3. Monolayer cultures of SK-N-MC were serum starved for 24 h and then treated for varying lengths of time with CLC or CNTF. As shown in Fig. 5C, both CLC and CNTF induce a rapid and sustained tyrosine phosphorylation of STAT-1. However under the same conditions, we did not detect any phosphorylation of STAT-3. These data suggest that CLC may be selective in the recruitment of STAT proteins.

DISCUSSION

In this study, a survey of a large EST database for putative secreted proteins yielded over 12,000 candidate cDNAs. Full-length sequencing of these cDNAs revealed a novel member of the neuropoietic cytokine family, CLC. The gene is widely expressed in adult human tissues; in particular, the spleen and peripheral blood leukocytes showed the highest level of expression. The gene maps to chromosome 11q13.3. The 22-kDa protein was expressed in *E. coli* and purified to near homogeneity. CLC induced the release of SEAP from TF-1, M-1 and U937 cells containing SRE- or NFκB-reporter constructs. Furthermore, CLC induces the tyrosine phosphorylation of gp130 and STAT-1 in the neuroblastoma cell line SK-N-MC.

Since the identification of CLC was based on a search strategy incorporating 2 algorithms designed to predict signal sequences, it is probable that the protein is secreted through the typical Golgi exocytotic pathway. Data supporting this finding were obtained from experiments in which CLC was identified in conditioned medium obtained from a CHO cell line stably transfected with CLC. In this regard, the structure of CLC is different from that of CT-1 or CNTF, which do not have canonical signal sequences and are thought to be released from cells via alternate pathways (28, 36). It is of interest to note that amino acid residues 135–160 represent an additional series of hydrophobic residues near the carboxy terminal end of the protein. CT-1 also has a hydrophobic region of similar size in relatively the same region of the protein. Although LIF has a similar hydrophobic region, it is less pronounced in comparison to CLC or CT-1. In contrast, CNTF and OSM do not have a comparable region in their sequence. Thus this second hydrophobic region is not a

common structural feature among the neuropoietic cytokines.

The result of the database screen demonstrated that CLC was present in a number of cDNA libraries including those obtained from activated or resting neutrophils, bone marrow stromal cells, and synovial fibroblasts. These data along with the results from the Northern analysis emphasize the higher level of expression in hematopoietic and immune related cell types implying that CLC may have a more selective action on these tissues. These findings are strengthened by the results of the experiments with the reporter cell lines. For example, TF-1 is a promyeloid cell line that is dependent on GM-CSF or IL-3 for long-term growth and provides a good *in vitro* system for investigating the proliferation and differentiation of myeloid progenitor cells.

Neuropoietic cytokines activate members of the Jak (1 and 2) and Tyk family of soluble tyrosine kinases that subsequently phosphorylate STAT proteins (37). The neuropoietic cytokines induce the phosphorylation of a subset of the six known STAT family members. CNTF and CT-1 induce the phosphorylation of STAT-3 in the neuroblastoma cell line SK-N-MC (7, 38). In primary cultures of chick ciliary ganglion cells, CNTF activates STAT-1 and -3. IL-6 and LIF activate STAT-5a in M1 myeloid leukemia cells (7, 39). When tested on the SK-N-MC cell line, CLC induces the tyrosine phosphorylation of STAT-1 but not STAT-3. In contrast, Robledo *et al.* also using SK-N-MC cells observed that CT-1 induces the tyrosine phosphorylation of STAT-3 but not STAT-1. Thus even though CT-1 and CLC are the closest family members based on sequence homologies, these two factors may activate different transcription regulatory pathways.

The identification of a novel member of the neuropoietic cytokine family documents the utility of a high-throughput screening effort to identify novel secreted factors in the HGS database. The hematopoietic and immune tissue expression pattern and the biological activity of CLC on a variety of related cell lines suggest that CLC may have an important regulatory function on these tissues either during development or in the adult.

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