Expression of biologically active mouse ciliary neutrophic factor (CNTF) and soluble CNTFRα in *Escherichia coli* and characterization of their functional specificities

Isabelle Cognet[1], Florence Guilhot[1], Sylvie Chevalier[2], Angélique Guay-Giroux[1], Alexandra Bert[1], Greg CA Elson[3], Hugues Gascan[2], Jean-François Gauchat[1]

[1] Département de pharmacologie, Université de Montréal, Montreal, Canada
[2] INSERM U564, CHU Angers, 4, rue Larrey, 49033 Angers, France
[3] NovImmune SA, Geneva, Switzerland

Correspondence: Jean-François Gauchat, Faculté de médecine, Département de pharmacologie, Université de Montréal, C.P. 6128, succursale Centre-ville, Montréal QC H3C 3J7. Fax: + 1 514 343 2291.
E-mail: jf.gauchat@umontreal.ca

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ABSTRACT. Ciliary neurotrophic factor (CNTF) is a neuroprotective cytokine initially identified in chick embryo. It has been evaluated for the treatment of neurodegenerative diseases. CNTF also acts on non-neuronal cells such as oligodendrocytes, astrocytes, adipocytes and skeletal muscles cells. CNTF has regulatory effects on body weight and is currently in clinical trial for the treatment of diabetes and obesity. CNTF mediates its function by activating a tripartite receptor comprising the CNTF receptor α chain (CNTFRα), the leukemia inhibitory factor receptor β chain (LIFRβ) and gp130. Human, rat and chicken CNTF have been expressed as recombinant proteins, and most preclinical studies in murine models have been performed using rat recombinant protein. Rat and human CNTF differ in their fine specificities: in addition to CNTFR, rat CNTF has been shown to activate the LIFR (a heterodimer of LIFRβ and gp130), whereas human CNTF can bind and activate a tripartite receptor comprising the IL-6 receptor α chain (IL-6Rα) and LIFR. To generate tools designed for mouse models of human diseases; we cloned and expressed in *E. coli* both mouse CNTF and the CNTFRα chain. Recombinant mouse CNTF was active and showed a high level of specificity for mouse CNTFR. It shares the arginine residue with rat CNTF which prevents binding to IL-6Rα. It did not activate the LIFR at all concentrations tested. Recombinant mouse CNTF is therefore specific for CNTFR and as such represents a useful tool with which to study CNTF in mouse models. It appears well suited for the comparative evaluation of CNTF and the two additional recently discovered CNTFR ligands, cardiotrophin-like cytokine/cytokine-like factor-1 and neuropoietin.

Keywords: cytokines, ciliary neurotrophic factor, interleukin-6 family

INTRODUCTION

CNTF was identified in the chick embryo for its capacity to support the survival of ciliary ganglion neurons in eye tissues [1, 2]. It promotes the survival and differentiation of a broad spectrum of rodent neuronal cells such as peripheral, sensory, sympathetic and parasympathetic neurons [3-5]. CNTF also acts on rodent non-neuronal cells such as oligodendrocytes [6-9], astrocytes [10, 11] adipocytes [12,13] and skeletal muscles cells [14-16]. The neuroprotective properties of CNTF have lead to preclinical trials in primate models of neurodegenerative diseases such as amyotrophic lateral sclerosis (ALS) and Huntington's disease and clinical trials in ALS patients [17-23]. CNTF contributes to weight regulation in mouse models of obesity [24, 25]. It is currently being evaluated for the treatment of obesity and diabetes [26].

A mutation inactivating the CNTF gene is homozygous in 2% of certain human populations [27, 28]. This mutation has been associated with early onset of multiple sclerosis (MS) [29] and ALS in patients with mutations in the gene coding for superoxide dismutase 1 [30], further supporting the neuroprotective role of CNTF in humans.

CNTF is a member of the IL-6 family of haematopoietic cytokines, with a four alpha helix basic structure, comprising two long cross-over loops and one short loop [31]. CNTF lacks a signal peptide and is therefore a cytosolic protein released by injured or damaged cells [32].

Rabbit [33], rat [32], chicken [34] and human CNTF [35-37] recombinant proteins have been expressed and characterized. Rat [32], pig [38] and human [35-37] CNTF cDNAs code for proteins of 200 amino acids. Rabbit [33], mouse [39] and chicken [34] CNTF are slightly shorter, with 199, 198 and 195 amino acids respectively. The CNTF sequence has been conserved during mammalian evolution, with more than 70% amino acid homology between mouse, rat, rabbit, pig, and human primary sequences.

The CNTF receptor (CNTFR) comprises three subunits: CNTFRα, leukemia inhibitory factor β receptor (LIFRβ) and gp130 [40, 41]. CNTFRα is a glycosylphosphatidylinositol (GPI) - anchored protein, which can also be found in a soluble form (sCNTFRα) [42]. The binding of CNTF to membrane bound or soluble forms of CNTFRα leads to the recruitment and activation of two signal transducing receptor subunits, LIFRβ and gp130, which are shared with other members of the IL-6 family [43]. Human [40], chick [44, 45] and canine [46] CNTFRα chains have been cloned. CNTFRα is a highly conserved, 372 amino acid protein with more than 90% of amino acids homology between the mouse, human, rat and dog sequences.

Despite their high level of homology, human and rodent CNTF differ according to their receptor specificities. Whereas human CNTF has an absolute requirement for a tripartite receptor to bind to the membrane, rat CNTF can activate cells expressing only LIFRβ and gp130 [47]. Conversely, the membrane IL-6Rα can substitute CNTFRα to form a receptor for human but not rat CNTF [48]. This particular property of human CNTF has been mapped to Gln-63 [48], which is replaced by an arginine in rat [32] and mouse CNTF [39]. These fine changes in the receptor specificity of CNTF between different species and of the human CNTF-derivative Axokine [25, 49] complicate the interpretation of findings documented using human cell cultures, rodent models, or in clinical trials. For example, the protective role of CNTF in the experimental autoimmune encephalomyelitis (EAE) multiple sclerosis (MS) mouse model was deduced from observations made using $CNTF^{-/-}$ mice [50]. If mouse CNTF does not absolutely require the CNTFRα component for its function [47], this protective role could be mediated by a receptor complex which is not activated by human CNTF or its therapeutic derivative Axokine [47, 49].

We have generated tools to study the precise receptor specificity of mouse CNTF for biological activity. Murine CNTF (mCNTF) and CNTFRα chain (mCNTFRα) cDNAs were cloned, and the corresponding proteins expressed in *Escherichia coli* (*E. coli*). Mouse CNTF or its complex with sCNTFRα were analysed for their biological activities using mouse Ba/F3 cells stably transfected with the functional tripartite human CNTF receptor or the two human LIFR chains, gp130 and LIFRβ [51]. Mouse CNTF in conjunction with its soluble receptor was further tested using the LIFRβ and gp130 expressing mouse myeloid M1 cell line [47, 52]. A high degree of functional specificity of mCNTF for the mouse receptor alpha chain was observed. The results obtained using recombinant mCNTF also indicate that the mouse cytokine displays an increased receptor specificity compared to its rat counterpart. Recombinant mCNTF and mCNTFRα represent new tools with which to study the function of CNTF and its therapeutic applications using mouse models.

METHODS

Recombinant cytokines

hCNTF and hCNTFRα were purchased from R&D Systems (Cedarlane Laboratories, Homby, ON, Canada).

Expression of mouse CNTF and sCNTFRα

The cDNA coding for mCNTFRα was amplified by polymerase chain reaction (PCR) from mouse brain cDNA (Invitrogen, Burlington, ON, Canada) using the primers CTTATGACTGCTTCTGTCCCATGGG and TTTCAGATCAGGAGATTGTTGGCTG. The amplified cDNA was cloned in pCR4TOPO (Invitrogen) and fully sequenced using an ABI-PRISM 3100 AVANT genetic analyser (Applied Biosystems, Streetsville, ON, Canada). The fragment coding for soluble mCNTFRα (smCNTFRα) was amplified using the primers CTAGCTAGCTACACGCAGAAACACAGTCC. and CCGCTCGAGGCTGCCAAGCTCCCCAGGGTC.

The cDNA coding for mCNTF was amplified by PCR from the IMAGE cDNA clone 1429801 (Open Biosystems, Huntsville, AL, USA) using the primers CAGCCATGGCTTTCGCAGAGCAATCA and CGTCTCGAGCATTTGCTTGGCCCCAT. The smCNTFRα and mCNTF amplified cDNA fragments were cloned in the *E. coli* expression vector pET24d (Novagen, Madison, WI, USA), using the restriction enzymes *Nhe*I and *Xho*I or *Nco*I and *Xho*I, respectively, fully sequenced and transformed in *E. coli* BL21 (DE3) Star (Invitrogen). Single colonies were used to inoculate Luria Bertani Broth (LB) containing 50 µg/mL kanamycin. Overnight cultures were diluted 1/50 and grown at 37 °C or 20 °C until an O.D. 600 of 0.6 was reached. Expression of the recombinant proteins was induced by addition of isopropyl-1-thio-β-D-galactopyranoside (IPTG; final concentration 1 mM) for 4 h at 37 °C or 16 h at 20 °C. Bacteria were harvested by centrifugation at 2 200 *g* for 15 min, suspended in 50 mM Tris-HCl pH 8.0, 50 mM NaCl, 1 mM EDTA, 1 mM PMSF, 1 mg/mL lysozyme, incubated at room temperature for 30 min, and lysed by sonication. The bacterial lysates were cleared by centrifugation (15 min., 25 000 *g*) at 4 °C. The centrifugation pellets containing the inclusion bodies were suspended in 10 mM Tris-HCl pH 8.0, 100 mM NaH_2PO_4, 8 M urea and incubated for 4 h at room temperature or overnight at 4 °C. Aliquots of each fraction were analyzed by SDS-PAGE on 12% polyacrylamide gels followed by Coomassie blue staining [53].

Purification of mouse CNTF and sCNTFRα

Recombinant mCNTF was purified from the soluble fraction under native conditions by affinity chromatography on nickel nitriloacetic (NiNTA) agarose (Qiagen, Mississauga, ON, Canada). The bacterial lysates cleared by centrifugation were dialyzed against 50 mM NaH_2PO_4, 300 mM NaCl, 10 mM imidazole pH 8.0. The soluble fractions were loaded on a 1 mL NiNTA column, washed with 50 mM NaH_2PO_4, 300 mM NaCl, 20 mM imidazole pH 8.0 and eluted with 50 mM NaH_2PO_4, 300 mM NaCl, 500 mM imidazole pH 8.0. Aliquots of each fraction (5 µL) were analysed by SDS-PAGE. The fractions containing the purified mCNTF were pooled and dialyzed against phosphate-buffered saline (PBS) pH 7.4.

Soluble mCNTFRα was purified from bacterial inclusion bodies by affinity chromatography on NiNTA agarose under denaturing conditions. The urea-solubilized inclusion bodies were loaded on a 1 mL NiNTA column. The resin was washed with 100 mM NaH_2PO_4, 10 mM Tris-HCl pH 8.0, 8 M urea pH 6.3. Soluble mCNTFRα was eluted in 100 mM NaH_2PO_4, 10 mM Tris-HCl, 8 M urea

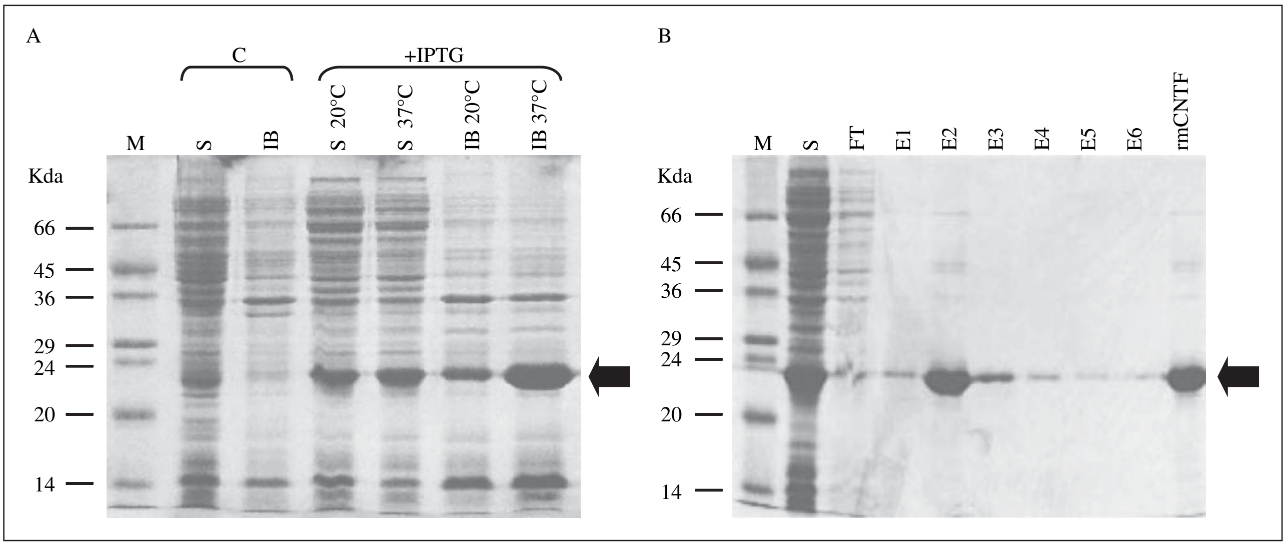

Figure 1

Expression and purification of mCNTF.
Panel A: Proteins were isolated from *E.coli* BL21 (DE3) Stars transformed with pET mCNTF before (lanes C), or after four and 16 hours of recombinant protein expression induction (lanes + IPTG) at 37 °C and 20 °C respectively. Lanes S: soluble fractions. Lanes IB: inclusion bodies. Aliquots representing equivalent amounts of bacteria were analysed by SDS-PAGE and Coomassie blue staining. Panel B. mCNTF was purified by IMAC from the cleared lysate of the bacteria induced at 37 °C, and the chromatography fraction was analysed by SDS-PAGE. Lane S: cleared lysate. Lane FT: flow through. Lanes E: elution fractions. Lane rmCNTF: purified mCNTF preparation after dialysis. Aliquots (5 µL) of the indicated fractions (S: 5 mL, FT: 30 mL, E: 500 µL, D: 1 ml) were analysed by SDS-PAGE and Coomassie blue staining. Panels A and B: Lane M: molecular mass marker with the size of the proteins indicated. The arrows show the migration of mCNTF.

pH 5.0 and analysed by SDS-PAGE. The mCNTFRα - containing fractions were pooled, and the protein was refolded by serial dialysis against PBS pH 8.0 containing decreasing concentrations of urea (7.5 M to PBS alone; 0.5 M steps). The precipitate formed during the dialysis was eliminated by centrifugation for 15 min. at 2 200 *g*.

Purified recombinant proteins were quantified using a BCA kit (Pierce, Biolynx, ON, Canada) and stored in aliquots at – 80 °C for further analysis.

Biological assay for mCNTF and mCNTFRα functional activity

Ba/F3 cells transfected with hgp130 and hLIFRβ or hgp130, hLIFRβ and hCNTFRα, (a kind gift from Dr. K.J. Kallen, Christian Albrechts, Universität zu Kiel, Germany) were cultured as described [51]. To test for mCNTF and mCNTFRα biological activities, Ba/F3 transfectants (10^4 cells/well in 96 - well plates) were incubated in triplicates with indicated dilutions of the recombinant proteins for 72 h in RPMI 1 640 supplemented with 10% foetal calf serum (FCS). [^{3} H] thymidine (20-30 Ci/mmol; Amersham Biosciences, Baie d'Urfé, QC, Canada) was added for 4 h and the incorporated radioactivity measured by scintillation counting.

M1 cells (ATCC, Manassas, VA, USA) were maintained in RPMI 1640 medium supplemented with 10% FCS. For the differentiation assays, M1 cells were incubated for 48 hours with the recombinant cytokines, stained for 30 min with FITC-labelled F4/80 monoclonal antibody (10 µg/mL; Caltag, Cedarlane Laboratories), and the fluorescence was analysed using a FACScan flow cytometer (BD biosciences, Mississauga, ON, Canada).

RESULTS

Expression and purification of recombinant mCNTF and mCNTFRα

Mouse CNTF cDNA was amplified from mouse brain first-strand cDNA and cloned in the expression vector pET24d under the control of the T7 polymerase promoter [54]. Recombinant mCNTF was expressed as a carboxy-terminal six histidine tagged protein in the *E. coli* derivative BL21 (DE3) Star at 20 or 37 °C *(Figure 1A)*. A high level of recombinant protein was detected in the soluble fractions and the inclusion bodies by growing the bacteria at either 20 or 37 °C, as assessed by SDS-PAGE analysis and Coomassie blue staining *(Figure 1A)*. The recombinant protein was purified from the soluble fraction by immobilized metal-ion affinity chromatography [55] *(Figure 1B)*. The cytokine-containing fractions (E2 in the experiment shown in *Figure 1*) were pooled and dialysed against PBS before being re-analyzed by SDS PAGE and Coomassie blue staining (*Figure 1B*, lane rmCNTF).

The fragment of mCNTFRα cDNA coding for the mature protein up to serine 342 (smCNTFRα) [40] was cloned in pET24d, in fusion with a carboxy-terminal six-histidine tag. Upon induction of the T7 polymerase promotor, production of the recombinant protein in *E. coli* BL21 (DE3) was readily detectable (*Figure 2A*, lanes IB). Most of the smCNTFRα was recovered in the insoluble fractions of the bacterial lysates containing the inclusion bodies, even when expression was performed at 20 °C, a condition known to increase recombinant protein solubility in *E. coli* [56] (*Figure 2A*, lanes IB). To isolate the recombinant protein, the inclusion bodies were solubilized in 8 M urea, and smCNTFRα was purified under denaturing conditions *(Figure 2B)*. The fractions containing the recombinant

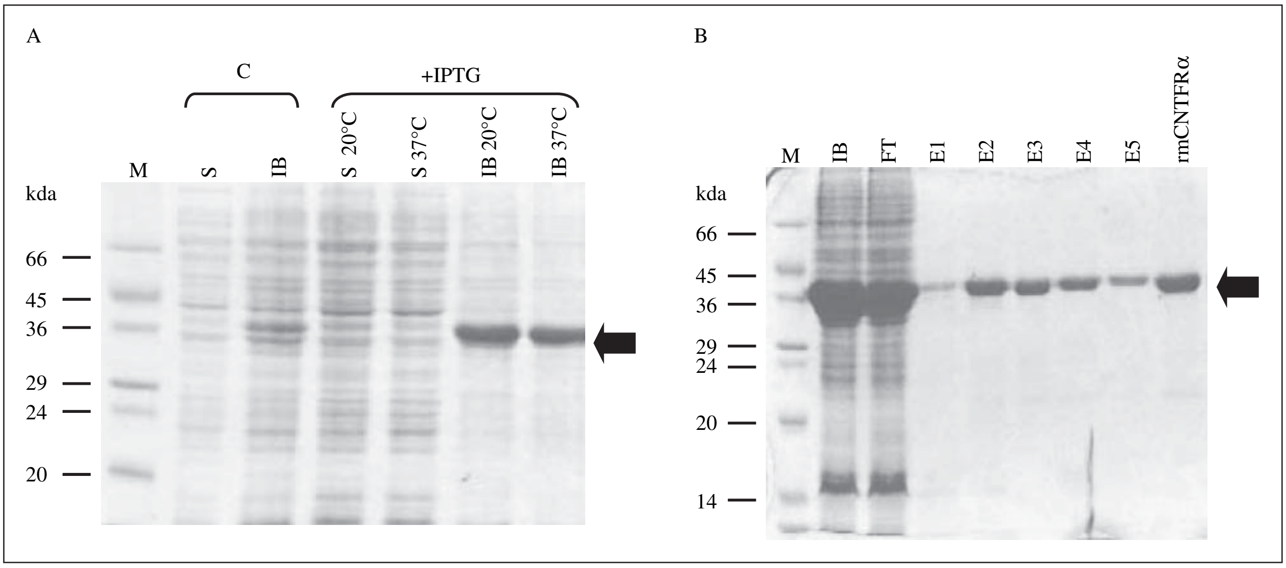

Figure 2

Expression and purification of smCNTFRα.

Panel A: Proteins were isolated from *E. coli* BL21 (DE3) Stars transformed with pET mCNTFRα before (lanes C), or after four and 16 hours of recombinant protein expression induction (lanes + IPTG) at 37 °C and 20 °C respectively. Lanes S: soluble fractions. Lanes IB: inclusion bodies. Aliquots representing equivalent amounts of bacteria were analysed by SDS-PAGE and Coomassie blue staining. Panel B. mCNTFRα was purified by IMAC from the insoluble fraction of the bacteria induced at 37°C and the chromatography fractions were analysed by SDS-PAGE. Lane IB: solubilized inclusion bodies. Lane FT: flow through. Lanes E: elution fractions. Lane rmCNTFRα: purified smCNTFRα preparation after refolding by step dialysis. Aliquots (5 µL) of the indicated fractions (S: 5 mL, FT: 30 mL, E: 500 µL, D: 1.5 mL) were analysed by SDS-PAGE and Coomassie blue staining. Panels A and B: Lane M: molecular mass marker with the size of the proteins indicated. The arrows show the migration of smCNTFRα.

proteins (corresponding to lane E2-E4 in the experiment shown in *Figure 2B*) were pooled and recombinant protein was refolded by slow dialysis against decreasing concentrations of urea to let the protein form the inter-strand disulfide bonds between the conserved cysteine residues of the receptor cytokine binding domain [40, 57]. Purified preparations of smCNTFRα were reanalysed by SDS-PAGE (*Figure 2B*, lane rmCNTFRα) and stored at – 80 °C.

Recombinant mCNTF induces the proliferation of Ba/F3 cells expressing human tripartite CNTF receptor

To assess the biological activity of the *E. coli* - expressed mCNTF, we used derivatives of the IL-3 - dependant cell line Ba/F3 rendered responsive to CNTF by transfection with the cDNA coding for the three subunits of the human CNTFR (hgp130, hLIFRβ and hCNTFRα) [51]. As expected from the high level of homology between mouse and human CNTF (82% amino acid homology), a strong proliferative response could be detected using the Ba/F3 expressing the human tripartite CNTF receptor (*Figure 3*, panel A). The proliferation observed was however reproducibly lower than the one induced by recombinant human CNTF (hCNTF; *Figure 3*, panel A).

The soluble form of the CNTFRα has been shown to form a complex with CNTF, which can activate the LIFR [42]. Therefore, to test the biological activity of the recombinant smCNTFRα, we assessed the proliferative response of Ba/F3 transfectants expressing LIFRβ and gp130 to the combination of smCNTFRα and hCNTF. A strong proliferation of the transfectants to the cytokine-soluble alpha chain complex was observed (*Figure 3*, panel B). The proliferation was only slightly lower than the one induced by the autologous shCNTFRα-hCNTF (*Figure 3*, panel B) complex.

Recombinant mCNTF shows a functional preference for the mouse alpha chain

To see if the lower potency of the recombinant mouse CNTF was due to a species specificity, we tested mCNTF in combination with the smCNTFRα expressed in *E. coli* BL21 (DE3) or shCNTFRα (*Figure 3*, panel C). Proliferation of the Ba/F3 cells expressing the functional LIFR (hLIFRβ and hgp130) was markedly higher in response to the mCNTF-smCNTFRα than in response to interspecies mCNTF-shCNTFRα hetero complex. The difference observed indicates a striking degree of specificity of mouse CNTF for the mouse receptor alpha chain (*Figure 3* panel C).

Interestingly, no proliferation of the Ba/F3 cells expressing the LIFR was detected in response to the recombinant mCNTF, even at the concentration of 250 pg/mL. This suggests a functional difference between mouse and rat CNTF. The latter has been shown to induce detectable proliferation of Ba/F3 transfectants expressing the human LIFR at concentrations above 10 pg/mL [47].

The recombinant mCNTF-mCNTFRα complex induces the differentiation of mouse myeloid leukemic M1 cells

To test the ability of mouse CNTF and CNTFRα to trigger the murine LIFR, we used the murine myeloid leukemic cell line M1. This cell line expresses mouse LIFRβ and gp130, and responds to LIF by growth arrest and an expression of macrophage differentiation markers [52, 58]. The mCNTF-mCNTFRα and hCNTF-hCNTFRα complexes were compared for their capacity to induce the

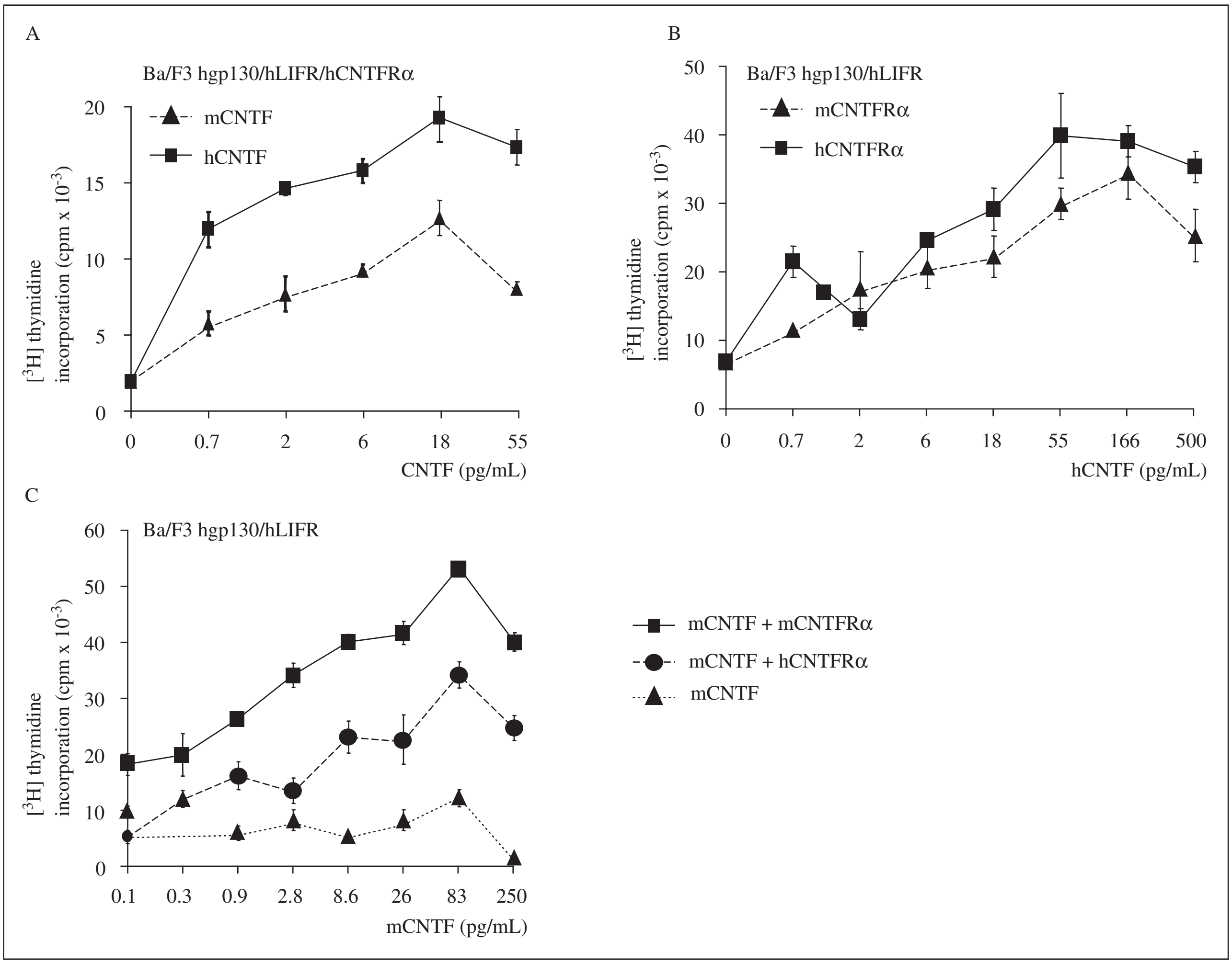

Figure 3

Panel A: mCNTF induces the proliferation of Ba/F3 cells transfected with hgp130, hLIFRβ and hCNTFRα. Panel B: smCNTFRα and hCNTF induce the proliferation of Ba/F3 cells transfected with hgp130 and hLIFRβ. Panel C: Comparison between the biological activities of mCNTF-smCNTFRα and mCNTF-shCNTFRα. Ba/F3 cells were stably transfected with the indicated receptor. cDNAs were cultured in the presence of serial dilutions of the indicated cytokines. When added, sCNTFRα was used at the optimal concentration of 2.5 µg/mL. Proliferation was measured by [^{3}H] thymidine incorporation, and experiments were performed in triplicate. Errors bars: standard errors of mean.

expression of the macrophage differentiation marker F4/80 [59, 60] in these cells. Maximal differentiation was observed at a concentration of 250 pg/mL of recombinant cytokine and 2,5 µg/mL of soluble receptor (*Figure 4* and data not shown). At all tested concentrations, the effect of mCNTF-mCNTFRα complex was indistinguishable from that observed for hCNTF-hCNTFRα (*Figure 4* and data not shown). Like its human homologue, mouse CNTF failed to induce detectable M1 differentiation in the absence of soluble CNTFRα chain (*Figure 4*, left panel), confirming the absolute requirement of the tripartite receptor to allow a functional response to mouse CNTF.

DISCUSSION

Using the *E. coli* BL21 (DE3) / pET system of T7 polymerase - driven recombinant protein expression controlled by the *lac* repressor [54], we were able to express both recombinant mouse CNTF and soluble CNTFRα. As reported previously for human CNTF [35], a large fraction of mCNTF was soluble in the bacterial extracts, and the recombinant protein could therefore be purified by IMAC under non-denaturing conditions.

Recombinant smCNTFRα was mostly detected in the insoluble fractions of the bacterial extracts containing the inclusion bodies. It was therefore solubilized using urea as a chaotropic agent, purified under denaturing conditions and refolded by step dialysis.

Mouse CNTF preparations were biologically active when tested for their capacity to induce the proliferation of the IL-3 - dependant cell line Ba/F3 transfected with the cDNAs coding for the human tripartite CNTFR receptor. Similarly, smCNTFRα could be shown to form a biologically active complex with commercial human CNTF when tested on Ba/F3 expressing the LIFR.

Purified mCNTF and smCNTFRα was able to form an active complex: this complex induced the proliferation of Ba/F3 cells expressing the human signal transducing subunits of the CNTFR (LIFRβ and gp130) or the differentiation of M1 cells expressing their mouse homologues.

When tested on Ba/F3 expressing the tripartite hCNTF receptor complex, mCNTF was significantly less potent than hCNTF. A comparison between the biological activities of mCNTF/ msCNTFRα and mCNTF/ hsCNTFRα complexes indicate that the reduced biological activity of mCNTF on Ba/F3 cells expressing the tripartite hCNTFR

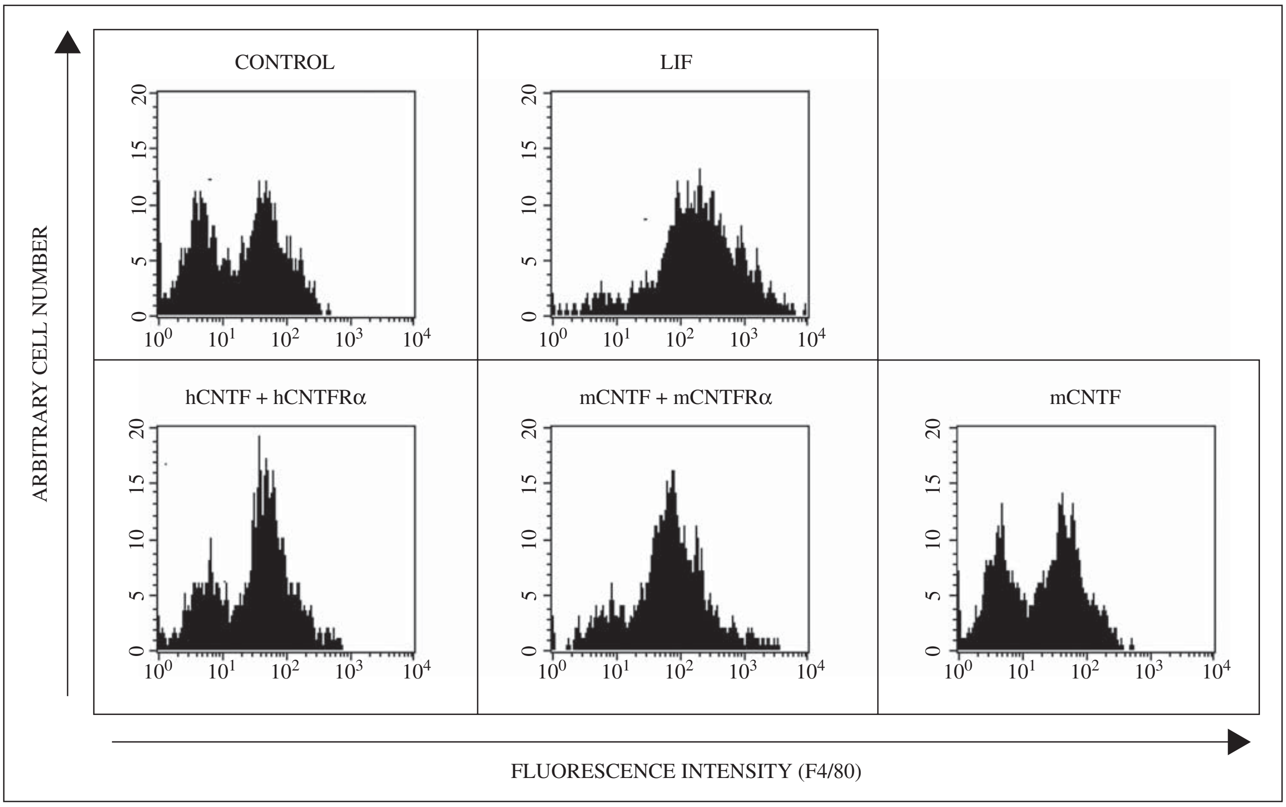

Figure 4
The mCNTF-smCNTFRα complex induces the expression of the differentiation marker F4/80 on LIFR expressing M1 cells.
Cells were incubated with the indicated cytokines (25 ng/mL) and soluble receptors (2,5 µg/mL) for 48 h and expression of the F4/80 marker was measures by flow cytometry.

could be explained by the specificity of mouse CNTF for its autologous receptor alpha chain, as observed previously with other cytokines.

Interestingly, mCNTF alone did not induce detectable biological activities on cells only expressing human or mouse LIF receptor complex. Mouse recombinant CNTF seems therefore to differ slightly from rat CNTF: rat CNTF has been shown to activate the LIFR in the absence of the CNTFRα chain at concentrations above 10 pg/mL [47]. Like rat CNTF, the mCNTF primary sequence comprises an Arg at the position corresponding to human Gln-63. This Arg has been shown to prevent binding of rat CNTF to the IL-6Rα chain [48]. Accordingly, we did not detect any biological activity of mCNTF preparations on the IL-6 - responsive M1 cells [60, 61] in the absence of the CNTFRα chain, even if the highest concentrations tested (250 pg/mL) were in the range in which human CNTF has been reported to activate a composite receptor comprising IL-6Rα, gp130 and LIFRβ [48].

As mCNTF did not show any biological activity on LIF receptor complex - expressing cells in the range of concentrations tested (up to 250 pg/mL), our results suggest that the protective functions of CNTF in mouse models such as EAE indicated by experiments with CNTF$^{-/-}$ mice [50], are mediated by the activation of a tripartite CNTFR comprising a soluble or membrane bound form of CNTFRα rather than due to a LIF – or IL-6 – like activity of mCNTF. The protective functions of mCNTF are therefore likely to be shared with human CNTF.

We have recently shown that CLC/CLF and neuropoietin are two new ligands for the CNTFR [62, 63]. The *E. coli* - produced recombinant mCNTF and smCNTFRα represent additional tools for the comparison of the therapeutic potential of CNTF with these more recently discovered CNTFR ligands using mouse models of neurodegenerative diseases or obesity. Recombinant mCNTF and mCNTFRα will also facilitate the generation of specific monoclonal antibodies.

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