Expression of a fusion protein of human ciliary neurotrophic factor and soluble CNTF-Receptor and identification of its activity *

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Abstract: Ciliary neurotrophic factor (CNTF) has pleiotropic actions on many neuronal populations as well as on glia. Signal transduction by CNTF requires that it bind first to CNTF-R, permitting the recruitment of gp130 and LIF-R, forming a tripartite receptor complex. Cells that only express gp130 and LIF-R, but not CNTF-R are refractory to stimulation by CNTF. On many target cells CNTF only acts in the presence of its specific agonistic soluble receptors. We engineered a soluble fusion protein by linking the COOH-terminus of sCNTF-R to the NH_2-terminus of CNTF. Recombinant CNTF/sCNTF-R fusion protein (Hyper-CNTF) was successfully expressed in COS-7 cells. The apparent molecular mass of the Hyper-CNTF protein was estimated from western blots to be 75 kDa. Proliferation assays of transfected BAF/3 cells in response to CNTF and Hyper-CNTF were used to verify the activity of the cytokines. The proliferative results confirmed that CNTF required homodimerization of the gp130, CNTF-R and LIF-R receptor subunit whereas Hyper-CNTF required heterodimerization of the gp130 and LIF-R receptor subunit. We concluded that the fusion protein Hyper-CNTF had superagonistic activity on target cells expressing gp130 and LIF-R, but lacking membrane-bound CNTF-R.

Key words: Ciliary neurotrophic factor(CNTF), Soluble CNTF-Receptor, Fusion protein, Biological activity

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INTRODUCTION

Ciliary neurotrophic factor (CNTF) is a naturally occurring protein with a molecular mass of approximately 22 kDa. It is expressed in glial cells within the central and peripheral nervous systems. CNTF stimulates gene expression, cell survival or differentiation in a variety of neuronal cell types such as sensory, sympathetic, ciliary and motor neurons (Ip *et al.*, 1991; Sendtner *et al.*, 1992; Larkfors *et al.*, 1994). The amino acid sequence of CNTF shows the features of a cytosolic protein with no signal peptide, no consensus sequences for glycosylation and only one free cysteine residue at position 17 (Sleeman *et al.*, 2000).

CNTF is structurally and functionally related to members of a family of cytokines that includes leukemia inhibitory factor (LIF), interleukin-6 (IL-6), interleukin-11, oncostatin M (OSM), cardiotrophin-1 (CT-1), and novel neurotrophin-1 (NNT-1)/cardiotrophin-like cytokine (CLC) (Taga *et al.*, 1997; Senaldi *et al.*, 1999). All IL-6 type cytokines use a membrane spanning 130-kDa glycoprotein, gp130, as a signal transducing receptor subunit. The biologic response to CNTF is elicited by formation of a multimeric receptor complex (Davis *et al.*, 1993b). CNTF first binds to a specific glycosyl-phosphatidylinositol-anchored α-unit, CNTF receptor (CNTF-R), which is not involved in signaling. This is followed by the recruitment of gp130 and LIF re-

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ceptor (LIF-R) as signal transducing β-units, which in turn form a disulfide-linked heterodimer that activates the JAK/STAT and the Ras/MAP kinase pathways (Taga *et al.*, 1997; Hirano, 1999). Cells, which only express signal transducing subunits but have no ligand binding subunits for these cytokines are refractory to stimulation.

In contrast to other known cytokine receptors, CNTF-R does not have a transmembrane or cytoplasmic region, but is anchored to the cell surface membrane by a glycosyl-phosphati-dylinositol linkage. The CNTF-R can function in either the membrane-bound form or soluble form, the latter being produced by phospholipase C-mediated cleavage of the membrane-bound receptor. The soluble CNTF-R has been detected in cerebrospinal fluid and serum, and has been shown to provide signaling capabilities to cells that are not highly responsive to CNTF alone (Davis *et al.*, 1993a).

Superagonistic cytokines consisting of covalently linked cytokines and soluble receptors have been designed. The first such molecule was Hyper-IL-6, a fusion protein in which IL-6 and soluble IL-6-R were connected by a flexible polypeptide linker(Fischer *et al.*, 1997). Hyper-IL-6 turned out to be fully active on cells expressing gp130 at concentrations 1/1000 to 1/100 that of unlinked IL-6 and sIL-6R. This approach had been adopted to obtain a superagonist of IL-11 and sIL-11R (Pflanz *et al.*, 1999).

We generated a CNTF/soluble CNTF-receptor (sCNTF-R) fusion protein (Hyper-CNTF) with superagonistic activity on target cells expressing gp130 and LIF-R, but lacking membrane-bound CNTF-R. In contrast to the existing cytokine/cytokine receptor fusion proteins Hyper-IL-6 and Hyper-IL-11 which directly activate the ubiquitously expressed gp130 protein, such a protein allows more specificity due to the restricted expression pattern of the LIF-R.

MATERIALS AND METHODS

1. Materials

Dulbecco's modified Eagle's medium (DMEM), penicillin, and streptomycin were purchased from Gibco (Karlsruhe, Germany). DEAE-Dextran was purchased from Sigma (Schnelldor, Germany). pcDNA3.1(-) expression vector was from Invitrogen (San Diego, CA). Restriction enzymes were from New England Biolabs (Frankfurt, Germany) and Fermentas (Heidelberg, Germany). [^{3}H]-thymidine (74 GBq/mmol) was obtained from Amersham Pharmacia Biotech UK (Buckinghamshire, England).

2. Cells and cytokines

DH5α was conserved by our laboratory. COS-7 cells, BAF/3-gp130/LIF-R cells and BAF/3-gp130 /LIF-R/CNTF-R cells (Kallen *et al.*, 1999) were grown in DMEM supplemented with penicillin (50 U/ml), streptomycin (50 μg/ml), and 10% foetal calf serum (FCS) at 5% CO_2 in water saturated atmosphere. BAF/3-gp130/LIF-R cells were cultured in the presence of 5 ng/ml human LIF, BAF/3-gp130/LIF-R/CNTF-R cells with 5 ng/ml human CNTF. Recombinant human CNTF was expressed in *Escherichia coli* strain BL21LysS as described (Kallen *et al.*, 1999). The concentration of human recombinant CNTF was determined using the human CNTF Quantikine Immunoassay Kit (R & D Systems, Wiesbaden, Germany). Recombinant human LIF were expressed in *E. coli* as described (Kallen *et al.*, 1999).

3. Construction of Hyper-CNTF expression plasmids

The cDNA sequences of human CNTF-R encoding the Ig-like domain and the cytokine binding domains (corresponding to amino acid residues 1-346) and human CNTF (corresponding to amino acid residues 1-186) were amplified by standard PCR technique. Using oligonucleotide primers, XbaI and SmaI restriction sites were introduced at the 5' and 3'-prime ends of the CNTF and CNTF-R cDNAs, respectively. The primer sequences used are available from the authors upon request. After digestion, both PCR products were ligated simultaneously into the XbaI site of the pcDNA3.1(-) expression vector. Ligation at SmaI led to the insertion of three additional nucleotides coding for glycine. The integrity of the construct was verified by restriction fragment analysis and DNA sequencing according to standard protocols (Sambrook *et al.*, 1989).

4. Extraction of plasmid DNA and restriction enzyme analysis of plasmid DNA were performed as described (Sambrook *et al*., 1989).

5. Purification of Hyper-CNTF plasmid was performed with QIAGEN-tip 500 Plasmid Kit (QIAGEN, Hilden, Germany) according to its protocol.

6. Expression of Hyper-CNTF in COS-7 cells

COS-7 cells were transiently transfected with plasmids coding for Hyper-CNTF by the DEAE-Dextran technique as described (Sambrook *et al*., 1989). Transfected cells were transferred to serum free medium after 24 hours and supernatants were collected on day 4 after transfection. The concentration of Hyper-CNTF was estimated by comparison of the intensity of immunoreactive bands of CNTF and Hyper-CNTF on the same Western blotting membrane using an anti-CNTF antiserum (Kruttgen *et al*., 1995).

7. Proliferation assays

BAF/3 cells stably transfected with expression vectors coding for human gp130 and human LIF-R (BAF/gp130/LIF-R) and human gp130, human LIF-R and human CNTF-R (BAF/gp130/LIF-R/CNTF-R) (Vollmer *et al*., 1999) were washed 3 times with PBS, and resuspended in cytokine free DMEM supplemented with 10% FCS at 5×10^3 cells/well in 96-well microtiter plates. Cells were cultured in a final volume of 100 μl with cytokines as indicated in the figure legends at 5% CO_2 in a water saturated atmosphere for 68 h and subsequently pulse labeled with 0.25 μCi [^{3}H]-thymidine for 4 h. Cells were harvested on glass fiber filters with a cell harvester system (Inotech, Dottikon, Switzerland). Incorporated [^{3}H]-thymidine was determined by scintillation counting (PerkinElmer, Freiburg, Germany). Proliferation assays were performed at least three times in triplicates for each cell line.

RESULTS

1. Construction of a fusion protein of CNTF/sCNTF-R

We engineered a soluble fusion protein by linking the COOH-terminus of sCNTF-R to the NH_2-terminus of CNTF. In principle, we followed the design of Hyper-IL-6 (Fischer *et al*., 1997) with two specific modifications. First, we included the NH_2-terminal Ig domain of the sCNTF-R since deletion of this region lead to reduced expression levels of recombinant sCNTF-RΔIg protein. This notice is in line with recent results indicating that the Ig-like domain of the IL-6R is important for intracellular transport of IL-6R through the secretory pathway (Vollmer *et al*., 1999). Secondly, we avoided the use of a synthetic polypeptide linker in order to minimize immunogenicity. Instead, the 16 C-terminal amino acids of CNTF-R (aa 331-346) which are not part of the membrane-proximal cytokine binding domain (Davis *et al*., 1991) and the 14 N-terminal non-helical and presumably flexible amino acids of CNTF (aa 1-12) (McDonald *et al*., 1995) were linked by one additional glycine residue. The resulting length of 31 amino acids, in analogy to Hyper-IL-6 and Hyper-IL-11, is presumably sufficient to connect both molecules and to allow access of CNTF to its CNTF-R binding site. A schematic model of the anticipated tertiary structure of the CNTF/sCNTF-R fusion protein is shown in Fig. 1. Agarose gel electrophoresis pattern of plasmid DNA of the CNTF/sCNTF-R fusion protein is shown in Fig. 2(in the next page).

2. Expression of the CNTF/sCNTF-R fusion protein

Transfected cells were transferred to serum free medium 24 hours after transfection of COS-7 cells with the Hyper-CNTF expression plasmid, and supernatants were collected on day 4 after transfection. Cleavage of the endogenous CNTF-R signal peptide in transfected COS-7 cells led to the secretion of the fusion protein Hyper-CNTF into the supernatant. Western blot analysis of Hyper-CNTF fusion protein from the supernatants of transfected COS-7 cells with an anti-CNTF antiserum is shown in Fig. 3a. The apparent molecular mass of the Hyper-CNTF protein was estimated from western blots to be 75 kDa. The concentration of Hyper-CNTF was estimated as 8 ng/μl by comparison of the intensity of immunoreactive bands of CNTF and Hyper-CNTF on the same Western blotting membrane using an anti-CNTF antiserum (Fig. 3b).

3. Biological activity of the CNTF/sCNTF-R fusion protein

Proliferation assays of transfected BAF/3

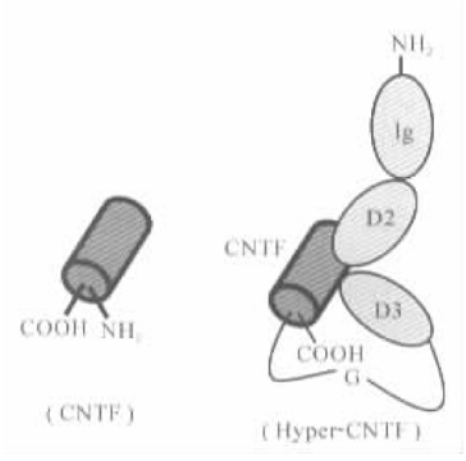

Fig.1 A schematic model of the anticipated tertiary structure of the CNTF/sCNTF-R fusion protein
The COOH-terminus of sCNTF-R was linked via one additional glycine residue (G) to the NH_2-teminus of CNTF. Ig denotes the immunoglobulin-like domain, D2 and D3 the two cytokine-binding receptor domains

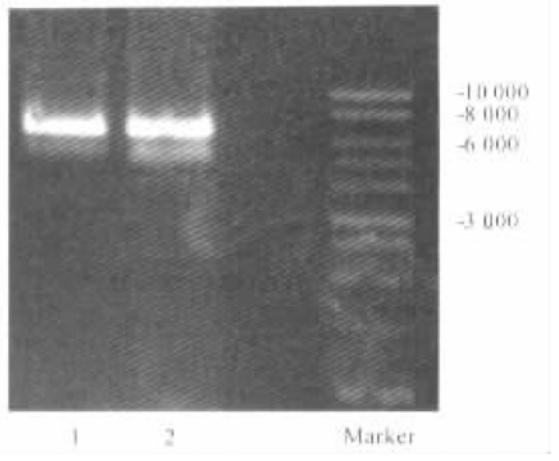

Fig.2 Agarose gel electrophoresis of plasmid DNA of the CNTF/sCNTF-R fusion protein (Hyper-CNTF) Marker: 1 kb DNA ladder; 1 and 2: restriction analysis of plasmid DNA of Hyper-CNTF with EcoRI-XbaI

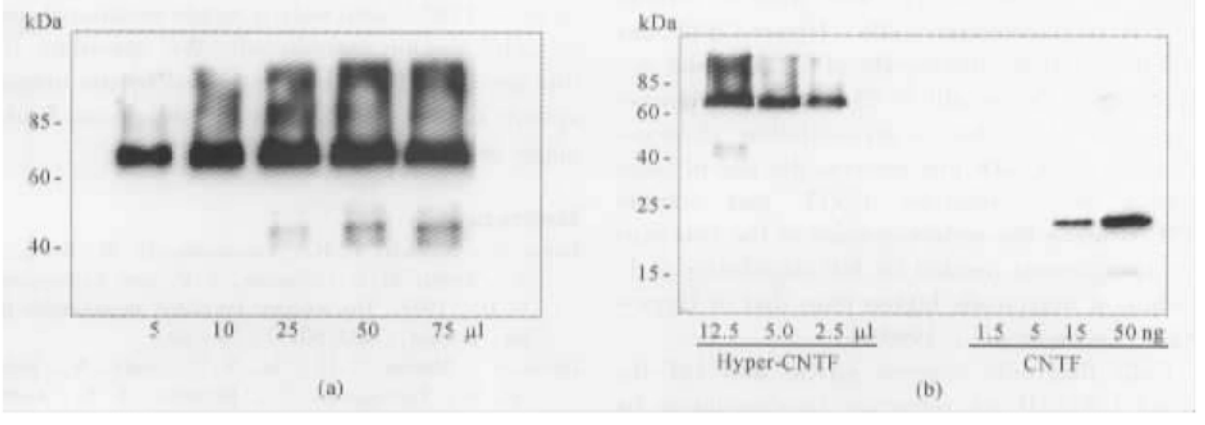

Fig.3 Western blot analysis of Hyper-CNTF expressed in COS-7 cells
(a) Western blot analysis of Hyper-CNTF from the supernatants of transfected COS-7 cells with an anti-CNTF antiserum; (b) The concentration of Hyper-CNTF was estimated by comparison of the intensity of immunoreactive bands of CNTF and Hyper-CNTF on the same Western blotting membrane using an anti-CNTF antiserum

cells in response to CNTF and Hyper-CNTF were used to verify the activity of the cytokines. BAF/3-gp130/LIF-R cells and BAF/3-gp130/LIF-R/CNTF-R cells were stimulated with increasing amounts of CNTF, Hyper-CNTF, LIF or medium alone. Proliferation of cells was assayed by measuring [^{3}H]-thymidine incorporation into DNA. As shown in Fig.4a, BAF/3-gp130/LIF-R cells proliferate upon stimulation with Hyper-CNTF and LIF but failed to respond to CNTF due to the lack of CNTF-R. In contrast, BAF/3-gp130/LIF-R/CNTF-R cells profilerated in response to CNTF, Hyper-CNTF and LIF (Fig.4b). The results confirmed that CNTF required homodimerization of the gp130, CNTF-R and LIF-R receptor subunit whereas Hyper-CNTF required heterodimerization of the gp130 and LIF-R receptor subunit (Taga *et al.*, 1997; Kallen *et al.*, 1999).

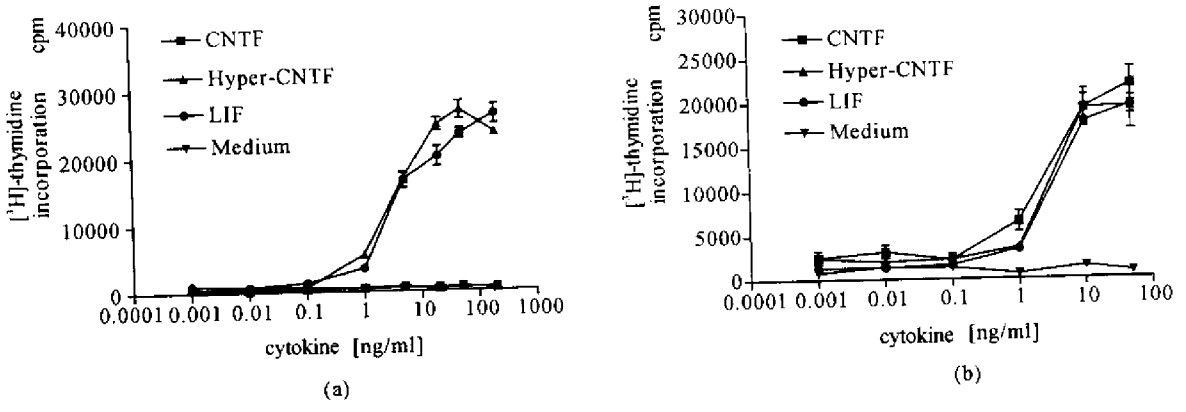

Fig.4 Proliferative assays of transfected BAF/3 cells to CNTF and Hyper-CNTF

(a) BAF/3-gp130/LIF-R cells and (b) BAF/3-gp130/LIF-R/CNTF-R cells were stimulated with increasing amounts of CNTF, Hyper-CNTF, LIF or medium alone. Proliferation of cells was assayed by measuring [^{3}H]-thymidine incorporation into DNA

DISCUSSION

We successfully expressed an active fusion protein of human CNTF and human soluble CNTF-R in mammalian cells. Hyper-CNTF has a calculated molecular weight of 65 kDA and apparent molecular weight of 75 kDa, the increase being most likely due to glycosylation. Expression of Hyper-CNTF circumvents the use of large amounts of recombinant CNTF and soluble CNTF-R since the concentrations of the two separate components needed for full stimulation is 1-2 orders of magnitude higher than that of Hyper-CNTF (Davis *et al*., 1993a).

Cells that only express gp130 and LIF-R, but not CNTF-R are refractory to stimulation by CNTF. As expected, BAF/3-gp130 /LIF-R cells lacking CNTF-R were responsive to Hyper-CNTF and LIF but not responsive to CNTF. Hyper-CNTF induced proliferation of BAF/3 cells expressing gp130 and LIF-R at virtually the same concentration as LIF.

The newly constructed Hyper-CNTF molecule has two main advantages over the Hyper-IL-6 and Hyper-IL-11 constructs. First, the spectrum of target cells is more restricted. All cells in the body express gp130 whereas only some cells including most cells of the nervous system express the LIF-R (März *et al*., 1999). Therefore, Hyper-CNTF seems to be more suited for *in vivo* application than Hyper-IL-6. Secondly, the fusion protein Hyper-CNTF does not contain a synthetic polypeptide linker, the CNTF-R and CNTF being linked via the flexible COOH-terminal portion of the CNTF-R and the NH_2-terminal part of CNTF (Davis *et al*., 1991; McDonald *et al*., 1995) with only a single additional amino acid residue introduced. We speculate that this protein will not be recognized by the immune system as a foreign protein and should not lead to major immune responses.

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