Human ciliary neurotrophic factor: a structure–function analysis

Alex KRÜTTGEN,*‡ Joachim GRÖTZINGER,* Günther KURAPKAT,* Joachim WEIS,† Ralph SIMON,† Michael THIER,† Michael SCHRÖDER,† Peter HEINRICH,* Axel WOLLMER,* Mike COMEAU,*§ Jürgen MÜLLBERG*§ and Stefan ROSE-JOHN*||

Institutes of *Biochemistry and †Neuropathology, RWTH Aachen, Pauwelsstrasse 30, D-52057 Aachen, Germany

Ciliary neurotrophic factor (CNTF) promotes survival *in vitro* and *in vivo* of several neuronal cell types including sensory and motor neurons. The primary structure of CNTF suggests it to be a cytosolic protein with strong similarity to the α-helical cytokine family which is characterized by a bundle of four anti-parallel helices. CNTF exerts its activity via complexation with CNTF receptor (CNTF-R). This complex consists of a CNTF-binding protein (CNTF-R) and two proteins important for signal transduction [gp130 and leukaemia inhibitory factor receptor (LIF-R)]. We have shortened the cDNA coding for CNTF at both the 5′ and the 3′ end and expressed the truncated proteins in bacteria. Biological activities of the protein preparations were determined by their ability to induce proliferation of BAF/3 cells that were stably transfected with CNTF-R, gp130 and LIF-R cDNAs. CNTF proteins with 14 amino acid residues removed from the N-terminus were biologically active whereas the removal of 23 amino acids resulted in an inactive protein. In addition, 18 amino acid residues could be removed from the C-terminus of the CNTF protein without apparent loss of bioactivity, but further truncation at the C-terminus yielded biologically inactive proteins. The introduction of two point mutations into the CNTF protein at a site that presumably interacts with one of the two signal-transducing proteins resulted in a CNTF mutant with no measurable bioactivity. In addition, a model of the three-dimensional structure of human CNTF was constructed using the recently established structural co-ordinates of the related cytokine, granulocyte colony-stimulating factor. CD spectra of CNTF together with our mutational analysis and our three-dimensional model fully support the view that CNTF belongs to the family of α-helical cytokines. It is expected that our results will facilitate the rational design of CNTF mutants with agonistic or antagonistic properties.

INTRODUCTION

Ciliary neurotrophic factor (CNTF) was first characterized as a survival factor of chick ciliary neurons [1]. It has been shown to exert neurotrophic activity on various neuronal cells including sensory and motor neurons [2,3]. Molecular cloning of CNTF cDNA showed that the protein consists of 200 amino acids. The lack of a signal peptide indicated that it is not secreted [4,5]. From the primary structure of CNTF and from secondary-structure predictions it has been postulated that the protein belongs to the α-helical cytokine family characterized by four anti-parallel α-helices. It shares considerable sequence homology with interleukin 6 (IL-6), leukaemia inhibitory factor (LIF), oncostatin M, and interleukin 11 [6,7] which together form the IL-6-type cytokine family [8].

The CNTF receptor (CNTF-R) is a glycosylphosphatidyl-inositol-anchored protein which is homologous to the IL-6R. The CNTF–CNTF-R complex interacts with two proteins, gp130 and LIF-R, which on interaction become linked via disulphide bridges [9–11]. The formation of the complex between CNTF, CNTF-R, gp130, and LIF-R leads to initiation of signal transduction [12]. Interestingly, the signal-transducing protein gp130 forms part of the receptor of all members of the IL-6-type cytokine family, suggesting that all these cytokines share an interaction site with gp130.

The secondary and tertiary structures of CNTF have not yet been determined and no detailed structure–function studies have been published. Preliminary results indicate that portions of the N- and C-termini of CNTF can be deleted without significant loss of bioactivity [13,14]. In the present study we have performed a detailed structure–function analysis of the CNTF protein based on the secondary-structure prediction of Bazan [7]. Furthermore, we have introduced point mutations into human CNTF at a site that in the human IL-6 protein has been shown to be important for initiation of signal transduction [15]. Finally, using the recently established co-ordinates of the related cytokine granulocyte colony-stimulating factor (G-CSF) we have constructed a three-dimensional model of CNTF which will facilitate the rational design of mutants of this protein with altered characteristics.

MATERIALS AND METHODS

Chemicals

Restriction enzymes, *Nco*I, *Pst*I and *Hin*dIII, calf intestinal phosphatase, T4 DNA ligase and random-priming DNA-labelling kit were purchased from Boehringer-Mannheim (Mannheim, Germany). Vent DNA polymerase were obtained from NEB (Schwalbach, Germany) and T7 DNA polymerase sequencing kit from Pharmacia (Freiburg, Germany). [α-^{32}P]dATP, [[α-^{35}S]thio]dATP and [^{3}H]thymidine were purchased from

Abbreviations used: CNTF, ciliary neurotrophic factor; G-CSF, granulocyte colony-stimulating factor; IL-6, interleukin 6; LIF, leukaemia inhibitory factor; R, receptor.

‡ Present address: Department of Neurobiology, Stanford University, Palo Alto, CA 94305-5401, U.S.A.

§ Present address: Immunex Corporation, 51 University Street, Seattle, WA 98101, U.S.A.

|| To whom correspondence should be addressed. Present address: I. Med. Klinik, Abt. Pathophysiologie, Obere Zahlbacher Str. 63, D-55101 Mainz, Germany.

Amersham International. The bacterial expression plasmid pRSET 5d and the host bacteria BL21 pLysS were described by Schöpfer et al. [16]. Polyclonal antiserum against human CNTF was prepared by immunization of rabbits against recombinant human CNTF. Oligonucleotides were synthesized using a Pharmacia DNA synthesizer.

Construction of mutants

Three N-terminal-deletion mutants of human CNTF were constructed by PCR by standard methods [17]. Mutants N-14aa, N-23aa, N-32aa were produced using the following sense oligonucleotides: 5′-CATGCCATGCACCTCTGTAGCCGCTC-3′ (sense primer for N-14aa); 5′-CATGCCATGGCAAGGAAGATTGCTT-3′(sense primer for N-23aa); 5′-CATGCCATGGCTCTTACGGAATCCTA-3′(sense primer for N-32aa); each contained an *Nco*I site upstream of the start codon ATG. The anti-sense primer was 5′-GGGAAGCTTCTACATTTTCTTGTTG-3′ coding for the last four amino acids of wild-type human CNTF and the stop codon followed by a *Hin*dIII site. The resulting PCR products were subsequently digested with *Nco*I and *Hin*dIII and subcloned into an appropriately digested pRSET-hCNTF plasmid. To minimize expenditure of control sequencing of the PCR-generated products, we replaced the 3′ portions of our mutant cDNAs between the two *Hin*dIII sites with an appropriate fragment taken from pRSET-hCNTF. After plasmid amplification, the subclones were preparatively digested with *Hin*dIII and cut into two fragments. The vector containing the 5′ portions of the deleted human CNTF cDNAs was separated from the smaller 3′ portion by electrophoresis through 1% agarose gels, isolated from the gel and purified by using a Quiaex kit (Diagen, Hilden, Germany). The resulting constructs were verified by sequencing between the *Nco*I site and the first *Hin*dIII site as described by Sanger et al. [18].

The double point mutant K154E/W157P was generated by PCR using the following oligonucleotides as primers: the sense primer was 5′-CATGCCATGGCTTTCACAGAGCAT-3′containing an *Nco*I site followed by the start codon ATG and the codons for the first five amino acids of wild-type human CNTF. The anti-sense primer was 5′-AAGCTCCTGCAGCACCTTTAGGCCCCGCAGCTTCTCCTCAAAGAGACC-3′, containing CTC [glutamate (E)] instead of CTT [lysine (K)] at position 154 and CGG [proline (P)] instead of CCA [tryptophan (W)] at position 157, followed by a *Pst*I site which also occurs at this position in wild-type human CNTF cDNA. The PCR product was digested with *Nco*I and *Pst*I and subcloned into an appropriately digested pRSET-hCNTF. This subclone was digested with *Hin*dIII and the fragment containing the 3′ portion of the mutated human CNTF cDNA was isolated and subsequently ligated into an appropriately digested and dephosphorylated pRSET-hCNTF. The sequence between the first *Hin*dIII site and the *Pst*I site containing the altered codons at positions 154 and 157 was verified by sequencing.

The C-terminal-deletion mutants were created by PCR using the following oligonucleotides as primers: 5′-GGGAAGCTTCTAGTTGTTAGCAATATAA-3′ (anti-sense primer for C-3aa); 5′-GGGAAGCTTCTAATGAGAAGAAATGAAA-3′ (anti-sense primer for C-18aa); 5′-GGGAAGCTTCTAAATGAAACGAAGGTC-3′(anti-sense primer for C-21aa); 5′-GGGAAGCTTCTAAAGGTCATGGATGGA-3′(anti-sense primer for C-24aa); each contained a *Hin*dIII site after the stop codon (underlined). The sense primer was 5′-CATGCCATGGCTTTCACAGAGCAT-3′, containing an *Nco*I site upstream of the start codon ATG and the codons for the first five amino acids of wild-type human CNTF. The PCR products were digested with *Hin*dIII and subcloned into an appropriately digested and dephosphorylated pRSET-hCNTF. The subclones were preparatively digested with *Nco*I and *Pst*I and cut into two fragments. The vector containing the 3′ portion of the mutated human CNTF cDNAs was separated from the smaller 5′ portion by electrophoresis on a 1% agarose gel, purified using a Quiaex kit and subsequently ligated with an appropriate fragment derived from pRSET-hCNTF. The resulting constructs were verified by sequencing between the *Pst*I and the second *Hin*dIII site.

Preparation and quantification of mutant proteins

E. coli bacteria (strain BL21 pLysS) were transformed with the expression vector pRSET containing wild-type or mutated human CNTF cDNAs and grown at 37 °C to an A_{600} of approx. 0.7. The bacteria were induced to produce the recombinant proteins by incubation in 0.4 mM isopropyl thiopyranogalactoside. After 3 h, inclusion-body preparation and denaturation with 6 M guanidinium chloride was performed as previously described [19]. Refolding was achieved by subsequent dialysis against 1 M guandinium chloride (12 h), 10% acetic acid (12 h) and double-distilled water (three times, 3 h each). The purity of the recombinant proteins was determined by SDS/PAGE (15% gels) and silver staining as well as by immunoblotting with a polyclonal antiserum against human CNTF (Figures 1c and 1d). Recombinant proteins were isolated to more than 90% purity. Protein concentration was determined by protein dye binding (Bio-Rad). In addition, the four most important proteins in this study (wild-type) human CNTF; N-23aa; C-24aa; K154E/W157P were hydrolysed in 6 M HCl and subjected to amino acid analysis to determine protein concentration.

Proliferation assay

Construction of BAF/3 cells transfected with cDNAs coding for human CNTF-R, human LIF-R and human gp130 has been described elsewhere [20]. These cells proliferate in response to human CNTF. Stably transfected BAF/3 cells were cultured in 96-well microtitre plates (1×10^4 cells/well; 0.2 ml/well) with the test samples for 72 h and pulse-labelled with [^{3}H]thymidine (0.5 μCi/well) for 5 h. To determine proliferation rates, cells were harvested on glass filters, and incorporated radioactivity was measured by scintillation counting. For each mutant, the bioassay was performed three times independently.

CD spectroscopy

CD spectra were recorded on an AVIV CD spectrometer 62DS and on a Jasco J-600 spectropolarimeter. Both instruments were calibrated with an aqueous solution of 10-camphosulphonic acid at 25 °C. The spectral bandwidth was 1.5 nm. Protein samples were dissolved in water and the pH was adjusted to 3.5 with small amounts of acetic acid. Protein concentrations were estimated by absorption spectroscopy at 278 nm. The CD spectrum in the far-UV was analysed with respect to the main-chain conformation of CNTF with the CONTIN program [21].

Molecular modelling

The model of CNTF was based on the X-ray structure of G-CSF [22], which served as a template. The sequential alignment was performed with the HUSAR program (EMBL, Heidelberg, Germany). For manipulation and graphic representation of the structures, the programs Whatif [23] and Chemx (developed and

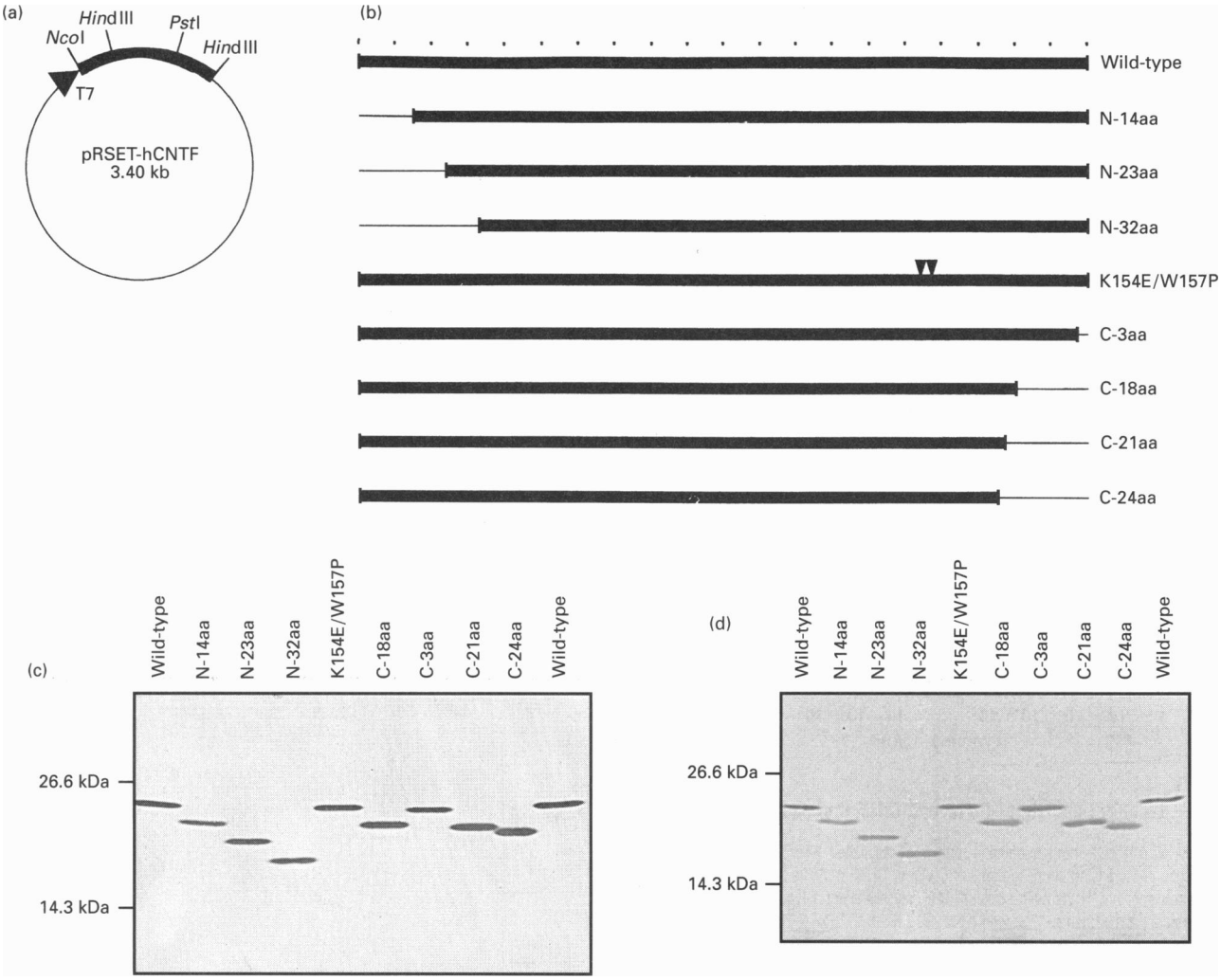

Figure 1 Expression of human CNTF and CNTF mutants

(**a**) Bacterial expression vector pRSET-hCNTF. (**b**) Schematic representation of the CNTF mutants constructed. (**c**) SDS/PAGE of the purified CNTF proteins. The proteins were visualized by silver staining. (**d**) Western blot of the mutants. The CNTF proteins were detected with a polyclonal CNTF antiserum raised against recombinant CNTF.

distributed by Chemical Design Ltd., Oxford, U.K.) running on an Indigo SGI and an Evans and Sutherland picture system 350 connected to a μVax respectively were used. Energy minimization and molecular-dynamics simulations were performed with the Gromos program library (W. F. van Gunsteren; distributed by BIOMOS biomolecular Software b.v., Laboratory of Physical Chemistry, University of Groningen, The Netherlands) for vacuum conditions. The parts of the CNTF model corresponding to the loop regions, which are missing in the G-CSF structure (G-CSF residues 65–70, 127–136, and 173–174), were generated by distance-geometry calculations and loop-search algorithms included in the software packages mentioned above.

RESULTS AND DISCUSSION

A CNTF expression vector was constructed by PCR technology with human CNTF genomic DNA as a template with a mixed oligonucleotide covering the end of exon I and the initial portion of exon II (Figure 1). On induction, bacteria expressed human CNTF at approx. 50% of the total cellular protein (results not shown). After recovery from inclusion bodies and refolding, recombinant CNTF was used for immunization of rabbits to produce a CNTF antiserum.

To determine which regions of the CNTF protein are important for biological function, we deleted portions of the CNTF cDNA at the 5′ and 3′ ends and obtained expression vectors for truncated CNTF proteins. The CNTF deletion mutants are schematically depicted in Figure 1(b). The proteins were expressed in bacteria, refolded and purified to homogeneity as shown in Figure 1(c). All CNTF mutants were recognized by the antiserum obtained by injecting recombinant CNTF into rabbits (Figure 1d).

We tested the ability of recombinant CNTF as well as mutated CNTF proteins to induce proliferation of BAF/3 cells stably transfected with expression vectors coding for human gp130, human CNTF-R and LIF-R, for which responsiveness to CNTF has been shown by Gearing et al. [20]. Figure 2(a) shows that truncation of the N-terminus by 14 amino acid residues had no effect on the biological activity of CNTF. Removal of 23 or 32 amino acids, however, completely abolished the capacity of CNTF to induce proliferation of transfected BAF/3 cells. When 3 or 18 amino acid residues were removed from the C-terminus

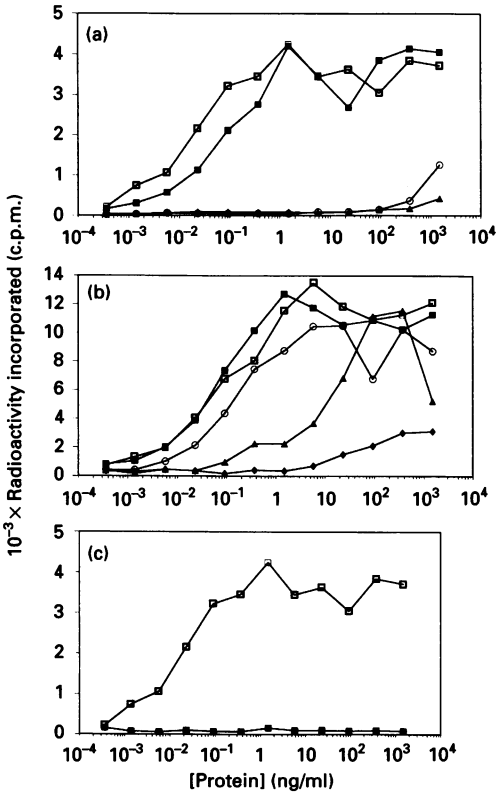

Figure 2 Biological activity of CNTF and CNTF mutants

(**a**) Biological activity of N-terminal-deletion mutants. Transfected BAF/3 cells were incubated in the presence of increasing concentrations of CNTF or CNTF mutants and proliferation of the cells was measured by [^{3}H]thymidine uptake. The data represent a typical result of one from three independent experiments. □, Wild-type; ■, N-14aa; ○, N-23aa; ▲, N-32aa. (**b**) Biological activity of C-terminal–deletion mutants measured as in (**a**). The data represent a typical result of one from three independent experiments. □, Wild-type; ■, C-3aa; ○, C-18aa; ▲, C-21aa; ◆, C-24aa. (**c**) Biological activity of the CNTF double mutant K154E/W157P measured as in (**a**). The data represent a typical result of one from three independent experiments. □, Wild-type; ■, K154E/W157P.

Table 1 Comparison of EC_{50} values determined for human CNTF and CNTF mutants

BAF/3 cells stably transfected with cDNAs coding for human CNTF-R, human LIF-R and human gp130 were grown in 96-well microtitre plates (1×10^4 cells/well, 0.2 ml/well) in the presence of human CNTF or CNTF mutants for 72 h and pulse-labelled with [^{3}H]thymidine (0.5 μCi/well) for 5 h. Cells were harvested on glass filters and incorporated radioactivity was determined. EC_{50} values are means ± S.E.M. of three independent experiments. 'Not active' indicates 10000-fold less active than human CNTF.

CNTF	EC_{50} (pg/ml)
Wild-type	90 ± 45
N-14aa	85 ± 20
N-23aa	Not active
N-32aa	Not active
C-3aa	87 ± 21
C-18aa	189 ± 61
C-21aa	21443 ± 223
C-23aa	Not active

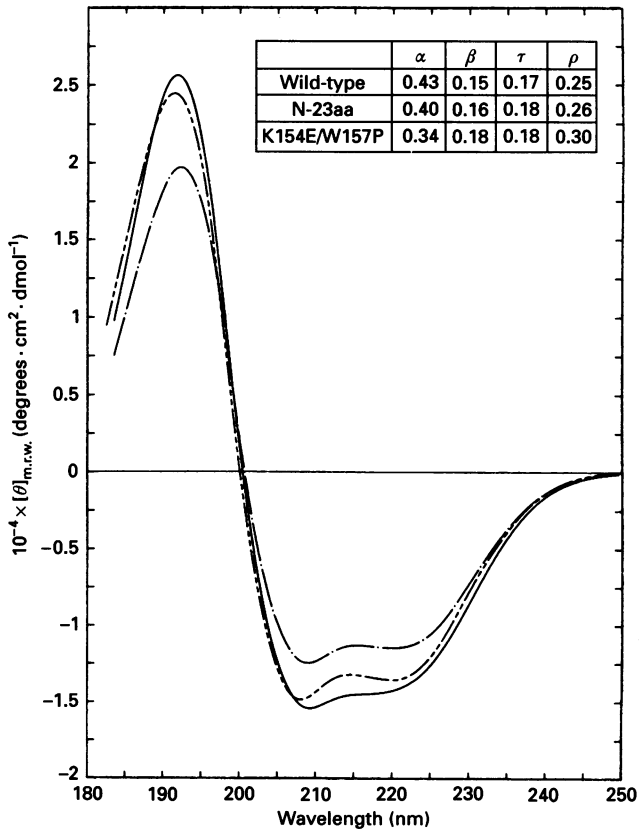

Figure 3 CD spectra of CNTF and CNTF mutants

CD spectra of recombinant human CNTF (——), N-23aa deletion mutant (– – –) and double point mutant (K154E/W157P) (–·–) in the far-UV. $[\theta]_{m.r.w.}$, Mean residue ellipticity. Inset: analysis of the far-UV CD spectra of recombinant human CNTF, N-23aa deletion mutant and double point mutant. α, α-helix; β, β-sheet; τ, turn; ρ, random coil.

of human CNTF, no significant change in biological activity was observed (Figure 2). Truncation of the protein by 21 residues reduced the bioactivity of CNTF by more than two orders of magnitude, and CNTF lacking 24 C-terminal amino acids was completely inactive. EC_{50} values of the CNTF mutants are shown in Table 1.

We have recently shown that the introduction of two point mutations at the top of the C-terminal helix of IL-6 renders the cytokine inactive on human hepatoma cells. This mutated IL-6 molecule with Glu-160 instead of Gln and Pro-163 instead of Trp turned out to be a partial IL-6R antagonist [15]. We therefore wondered whether the same point mutations introduced into the CNTF protein at the corresponding region would result in a similar phenotype. On the basis of molecular modelling results, we decided to introduce a glutamate at position 154 and a proline at position 157. As shown in Figure 2(c), the K154E/W157P mutant was completely inactive even when administered at doses that were about 10000-fold higher than the EC_{50} of human CNTF (see Table 1).

CD spectra in the far-UV region of the wild-type CNTF, the N-23aa deletion mutant and the K154E/W157P double mutant are shown in Figure 3. All three spectra are characteristic of proteins with a substantial helical content. Although the double mutant was biologically inactive, much of the original fold was retained.

The inset of Figure 3 shows the analysis of the spectra of three

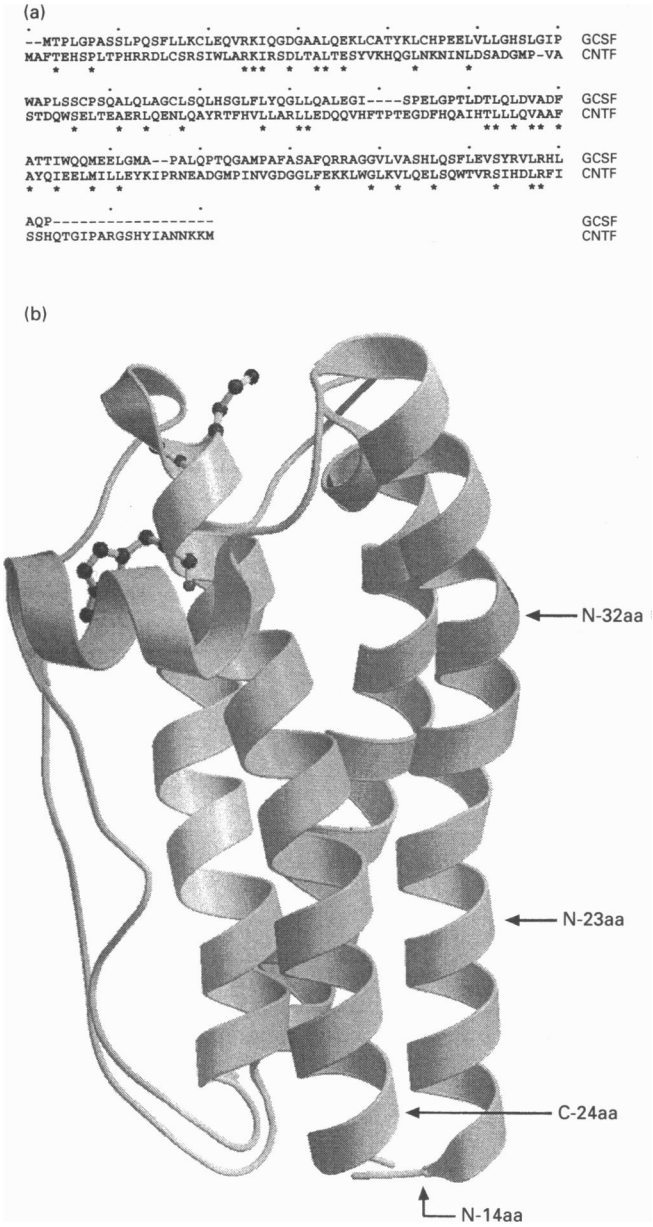

Figure 4 Molecular modelling of CNTF

(**a**) Sequence alignment of human G-CSF and CNTF; (**b**) Three dimensional model of human CNTF. The side chains of Lys-154 and Trp-157 and the positions of the deletions analysed in this study are shown.

molecules with respect to their main-chain conformation by the CONTIN program [21]. The helix content of the wild-type protein was in good agreement with the model depicted in Figure 4(b). The reduction in helix content of the N-23aa deletion and the K154E/W157P double mutant is compensated mainly by a corresponding increase in the amount of random coil, whereas the amounts of β-structure and turn remained unchanged.

The three-dimensional structure of CNTF is not known but recently the structure of G-CSF has been solved by X-ray crystallography [22]. G-CSF is one of the α-helical cytokines with marked similarity to human CNTF. We therefore used the co-ordinates of the G-CSF structure to build a model of the CNTF molecule. Figure 4(a) shows the alignment of the human CNTF sequence with the human G-CSF sequence. The result obtained by the multialignment program (EMBL) was changed partially to give a better agreement with the experimental structure of G-CSF [22]. The location of the helical parts was checked as follows. From a graphical inspection of the G-CSF structure, we deduced which amino acids are essential for building the hydrophobic core of the protein. This resulted in a pattern of hydrophobic residues that should be conserved in the CNTF structure. The above alignment was then corrected to give the best agreement with these patterns. The side chains of residues in G-CSF were exchanged for those of CNTF according to the alignment. Because there is no structural information available for the first eight amino acids of G-CSF and as CNTF has an N-terminal extension of three and a C-terminal extension of 18 amino acids, our model cannot describe these parts of the molecule.

A first inspection of the protein core showed fairly good packing of the molecule without unfavourable contacts between side-chain atoms. The structure was energy-minimized over 500 steps using the steepest-descent algorithm. Further refinement was achieved by molecular-dynamics simulation over a time span of 50 ps using a simulated annealing protocol followed by a 10 ps simulation in which the system was cooled down to 10 K to freeze the structure into a single conformation. A cartoon of this structure is shown in Figure 4(b). Arrows indicate the positions of the last amino acid of the N- and C-terminal deletion mutants constructed.

Figure 4(b) also shows the location of the K154E/W157P double mutant. Lys-154 is located at the beginning of helix D. The four methylene groups of its side chain cover a hydrophobic area of the molecule whereas the charged amino group is accessible to water molecules. From our model we deduced that shortening of the side chain (Lys to Glu) and the consequent placing of a charge on to a hydrophobic surface would lead to major structural changes in this region of the molecule. The replacement of Trp-157, which is located in the D-helix, with Pro should break the helix at this position. Moreover, the bulky side chain of Trp-157 is located in a hydrophobic environment to which residues from the small helix A′ contribute. Replacing this bulky side chain by the much smaller proline side chain should lead to gross perturbations in this hydrophobic cluster and should probably also alter the main-chain conformation of the small helix A. From the model we can predict that both effects, disturbance of the end of helix A and the small helix A′, should lead to substantial loss of helical content of the molecule which is in good agreement with the data derived from CD spectroscopy.

The results for the truncated CNTF proteins are reminiscent of data obtained with IL-6 proteins mutated in a similar fashion [24–29]. Truncation of the IL-6 protein by removal of more than 29 amino acids from the N-terminus abolished all biological activity whereas it required the removal of only three amino acids from the C-terminus to produce a biologically inactive protein. According to the model described for the CNTF structure, C-terminal truncation of CNTF by 18 amino acids only removes the non-helical terminal segment of the molecule, whereas further truncation removes the C-terminus of helix D. Surprisingly, a recent report states that a CNTF mutant lacking 28 amino acids from the C-terminus [14] was even more active than full-length human CNTF. The mutation strategy employed by these authors, however, involved the inclusion of plasmid sequences in the open reading frame of CNTF resulting in the translation of unrelated amino acid residues [14]. In the case of the CNTF mutant lacking 28 amino acids, the additional amino acid residues might have substituted for the natural CNTF C-terminus.

As in IL-6, the end of the C-terminal helix (helix D) of CNTF seems to be important for the structural integrity of the protein. A possible reason could be that, as in IL-6, the N-terminus of helix A and the C-terminus of helix D are in close proximity and could stabilize each other [28,30]. In the case of IL-6, the beginning of helix D has been shown to be involved in the interaction with the signal-transducing protein gp130 [15]. An IL-6 protein with the point mutations Q160E/W163P lacks biological activity on human hepatoma cells and exhibits antagonist activity [15]. The analogous point mutations introduced into the CNTF protein produced a mutant with a similar phenotype; CNTF K154E/W157P had no detectable biological activity even when applied at high concentrations, 10000-fold higher than the EC_{50} of human CNTF.

Taken together the results of these studies provide experimental proof for the structural model of Bazan [7] which predicted close structural similarity between IL-6 and CNTF. For IL-6 it has recently been shown that the regions at the beginning of helix A and at the end of helix D are involved in the specific recognition of the IL-6R whereas the region at the end of helix A and the beginning of helix D are involved in the interaction with the signal-transducing molecule gp130 [15,30]. In the case of CNTF it has been shown that the CNTF–CNTF-R complex interacts with a heterodimer of gp130 and LIF-R. It is therefore tempting to speculate that the region around the point mutations K154E/W157P are involved in the recognition of either gp130 or LIF-R.

It would be of interest to identify the exact points of contact between gp130 or LIF-R and the CNTF–CNTF-R complex, e.g. by introducing a set of point mutations into the first portion of helix D. This would help to explain how the cytokine, IL-6, interacts with a homodimer of gp130 whereas CNTF recruits a heterodimer of gp130 and LIF-R. Such information would greatly facilitate the rational design of CNTF antagonists and agonists with improved activity.

We thank Dr. F. Altruda (Torino, Italy) for the gift of human genomic CNTF clone and Dr. R. Laufer (IRBM, Pomezia, Italy) for immunization of rabbits. The gift of the transfected BAF/3 cell lines from Dr. D. Gearing (Systemix, Palo Alto, CA, U.S.A.) is gratefully acknowledged. We especially thank Dr. F. Bazan (DNAX, Palo Alto, CA, U.S.A.), Dr. J. Brakenhoff (CLB, Amsterdam, The Netherlands) and Dr. D. Cosman (Immunex, Seattle, WA, U.S.A.) for stimulating discussions and support. This work was supported by grants from the Deutsche Forschungsgemeinschaft to S. R.-J.

Received 25 October 1994/20 February 1995; accepted 3 March 1995

REFERENCES

1 Adler, R., Landa, K. B., Manthorpe, M. and Varon, S. (1979) Science **204**, 1434–1436
2 Skaper, S. D. and Varon, S. (1986) Brain Res. **389**, 39–46
3 Oppenheim, R. W., Prevette, D., Quin-Wei, Y., Collins, F. and MacDonald, J. (1991) Science **251**, 1616–1618
4 Lin, L.-F. H., Mismer, D., Lile, J. D. et al. (1989) Science **246**, 1023–1025
5 Stöckli, K. A., Lottspeich, F., Sendtner, M. et al. (1989) Nature (London) **342**, 920–923
6 Bazan, J. F. (1990) Immunol. Today **11**, 350–354
7 Bazan, J. F. (1991) Neuron **7**, 197–208
8 Mackiewicz, A., Koj, A. and Sehgal, P. B. (eds.) (1995) Ann. N.Y. Acad. Sci., vol. 762
9 Davis, S., Aldrich, T. H., Valenzuela, D. M. et al. (1991) Science **253**, 59–63
10 Davis, S., Aldrich, T. H., Stahl, N. et al. (1993) Science **260**, 1805–1808
11 Ip, N. Y., Nye, S. H., Boulton, T. G. et al. (1992) Cell **69**, 1121–1132
12 Stahl, N., Davis, S., Wong, V. et al. (1993) J. Biol. Chem. **268**, 7628–7631
13 Panayotatos, N., Radziejewska, E., Acheson, A., Pearsall, D., Thadani, A. and Wong, V. (1993) J. Biol. Chemn. **268**, 19000–19003
14 Negro, A., Corsa, V., Corona, G., Grandi, C., Skaper, S. D. and Callegaro, L. (1994) Neurochem. Res. **19**, 223–227
15 Brakenhoff, J. P. J., deHon, F. D., Fontaine, V. et al. (1994) J. Biol. Chem. **269**, 86–93
16 Schöpfer, R., Halvorsen, S. W., Conroy, W. G., Whiting, P. and Lindstom, J. (1989) FEBS Lett. **257**, 393–395
17 Sambrook, J., Fritsch, L. F. and Maniatis, T. (1989) Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY
18 Sanger, F., Nicklen, S. and Coulson, A. R. (1977) Proc. Natl. Acad. Sci. U.S.A. **74**, 5463–5467
19 Van Dam, M., Müllberg, J., Schooltink, H. et al. (1993) J. Biol. Chem. **268**, 15285–15290
20 Gearing, D. P., Ziegler, S. F., Comeau, M. R. et al. (1994) Proc. Natl. Acad. Sci. U.S.A. **91**, 1119–1123
21 Provencher, S. W. and Glöckner, D. J. (1981) Biochemistry **20**, 33–37
22 Hill, C. P., Osslund, T. D. and Eisenberg, D. (1993) Proc. Natl. Acad. Sci. U.S.A. **90**, 5167–5171
23 Vriend, G. (1990) J. Mol. Graph **8**, 52–56
24 Krüttgen, A., Rose-John, S., Möller, C. et al. (1990) FEBS Lett. **262**, 323–326
25 Krüttgen, A., Rose-John, S., Dufhues, G. et al. (1990) FEBS Lett. **273**, 95–98
26 Lütticken, C., Krüttgen, A, Möller, C., Heinrich, P. C. and Rose-John, S. (1991) FEBS Lett. **282**, 265–267
27 Brakenhoff, J. P. J., Hart, M. and Aarden, L. A. (1989) J. Immunol. **143**, 1175–1182
28 Brakenhoff, J. P. J., Hart, M., De Groot, E. R., Di Padova, F. and Aarden, L. A. (1990) J. Immunol. **145**, 561–586
29 Leebeck, F. W. G., Kariya, K., Schwabe, M. and Fowlkes, D. M. (1992) J. Biol. Chem. **267**, 14832–14838
30 Ehlers, M., Grötzinger, J., deHon, F. D. et al. (1994) J. Immunol. **153**, 1744–1753