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D1 cap region involved in the receptor recognition and neural cell survival activity of human ciliary neurotrophic factor

(mutagenesis/cytokine/glycoprotein gp130/antagonist)

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ABSTRACT **Human ciliary neurotrophic factor (hCNTF), which promotes the cell survival and differentiation of motor and other neurons, is a protein belonging structurally to the α-helical cytokine family. hCNTF was subjected to three-dimensional structure modeling and site-directed mutagenesis to analyze its structure–function relationship. The replacement of Lys-155 with any other amino acid residue resulted in abolishment of neural cell survival activity, and some of the Glu-153 mutant proteins had 5- to 10-fold higher biological activity. The D1 cap region (around the boundary between the CD loop and helix D) of hCNTF, including both Glu-153 and Lys-155, was shown to play a key role in the biological activity of hCNTF as one of the putative receptor-recognition sites. In this article, the D1 cap region of the 4-helix-bundle proteins is proposed to be important in receptor recognition and biological activity common to α-helical cytokine proteins reactive with gp130, a component protein of the receptors.**

Ciliary neurotrophic factor (CNTF), originally described as a trophic factor supporting the survival of ciliary ganglion neurons *in vitro* (1), has been well known to promote the survival and differentiation of motor neurons and other neuronal cells *in vitro* and *in vivo* (2–4). The effectiveness of treatment with CNTF in motor-neuron-degenerative animal models such as wobbler (5) and pmn mice (6) and in the facial-nerve axotomy model (7) led to a clinical trial targeted to patients suffering from the neuronal degenerative disease amyotrophic lateral sclerosis (8, 9). In spite of the detailed analysis and accumulated information on biological activity and efficacy, little is yet known about the tertiary structures, the structure–function relationship of CNTF, or the mode of interaction with its receptors.

CNTF functionally acts on neuronal cells as do neurotrophins (NTs) such as NT-3, NT-4/5, nerve growth factor, and brain-derived neurotrophic factor. However, it is greatly different from NTs in both the molecular structure and the mode of receptor interaction. Human CNTF (hCNTF) is a 200-amino-acid-residue protein rich in α-helical structure, having 53% α-helices and 9% β-turns (10). A secondary structure analysis suggested that CNTF had a structural topology similar to the α-helical cytokines, though the amino acid sequence of hCNTF had no homology with them. All α-helical cytokines such as growth hormone (GH), prolactin (PRL), erythropoietin (EPO), granulocyte colony-stimulating factor (G-CSF), leukemia inhibitory factor (LIF), and some interleukins (ILs) (11) have a common structure with a characteristic bundle of four antiparallel helices, A, B, C, and D. As for the receptor, CNTF binds to CNTF receptors (CNTFRs) consisting of CNTFR α, LIF receptor (LIFR), and gp130, the last of which is a common glycoprotein component among the receptors for CNTF, interleukin 6 (IL-6), LIF, oncostatin M, and interleukin 11 (IL-11) (12–14).

Both the AB loop region (amino acid residues between helix A and B) and helix D have been proposed so far to be important for the biological activity of α-helical cytokine family proteins such as IL-6 (15, 16). These regions of GH as well as IL-6 were predicted by a mutagenesis technique to be involved in the receptor binding (17). This was confirmed later by x-ray crystallography of the GH–receptor(s) complex (18). The boundary between the CD loop and helix D in α-helical cytokines is assumed to structurally serve as an N-terminal "cap" to helix D and is called the "D1 cap region" (11). In this region, the "D1 motif" consisting of ϕ-(Phe or Trp)-(Glu or Gln)-(Lys or Arg)$_2$-ϕ-Xaa-Gly (ϕ = hydrophobic residue and Xaa = any residue) is proposed as the consensus sequence. The D1 motif is commonly seen, with some exceptions, in the α-helical cytokine family (Table 1). This boundary region between the CD loop and helix D is also predicted to be important for receptor binding in the case of IL-6 (21) and LIF (22). A line of evidence described above suggested that the CNTF residues at the AB loop, helix D, and the D1 cap region might be involved in receptor recognition and/or structural stability.

Therefore, we analyzed the structure–function relationship of hCNTF by site-directed mutagenesis targeted on the amino acid residues in the region of the AB loop, helix D, and CD loop (especially the D1 cap region).

MATERIALS AND METHODS

Construction of *Escherichia coli* Expression Plasmid of hCNTF (pKKCNTF). The hCNTF gene was cloned from the human placenta DNA (Clontech) by the PCR method as described by Masiakowski *et al.* (23) with a modification. The PCR fragment encoding whole hCNTF was inserted into the *Bam*HI and *Pst* I site of pKK223-4 (Pharmacia).

Site-Specific Mutagenesis of hCNTF. A two-part PCR was used to create the mutation on pKKCNTF using three primers: p#N, 5′-CGGAGATCTTTTTTATAAAATCAGGAGG-3′ (at the 5′ end of the hCNTF gene; plus strand); p#C(RV), 5′-CTTGCATGCATGCATGTCAGAGAAGGGAC-3′ (at the 3′ end of the hCNTF gene; minus strand), and the primer corresponding to the mutating site. For example, pK155 (5′-CTTTGAGAANNNCTGTGGG-3′; plus strand) was used for the Lys-155 site, or pE153 (5′-GGTCTCTTTNNNAAGAAG-CTGTG-3′; plus strand) was used for the Glu-153 site; in these primers NNN indicates the codon corresponding to a substituting amino acid. These PCR products were subcloned into

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Abbreviations: CNTF, ciliary neurotrophic factor; CNTFR, CNTF receptor; hCNTF, human CNTF; IL, interleukin; IL-6 and IL-11, interleukins 6 and 11; DRG, dorsal root ganglion; LIF, leukemia inhibitory factor; LIFR, LIF receptor; GH, growth hormone; G-CSF, granulocyte colony-stimulating factor.

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Table 1. Amino acid sequences in D1 cap region of 4-helix-bundle proteins

Protein	Consensus sequence								Amino acid residue
	ϕ	Phe or Trp	Glu or Gln	Lys or Arg	Lys or Arg	ϕ	Xaa	Gly	
Subgroup IA									
hCNTF†	Leu	*Phe*	*Glu*	*Lys*	*Lys*	Leu	Trp	*Gly*	151–158
Rat CNTF*	Leu	*Phe*	*Glu*	*Lys*	*Lys*	Leu	Met	*Gly*	150–157
Rabbit CNTF*	Leu	*Phe*	*Glu*	*Lys*	*Lys*	Leu	Trp	*Gly*	151–158
Human LIF*	Val	*Phe*	*Gln*	*Lys*	*Lys*	Lys	Leu	*Gly*	154–161
Mouse LIF*	Ala	*Phe*	*Gln*	*Arg*	*Lys*	Lys	Leu	*Gly*	154–161
Human OSM*	Ala	*Phe*	*Gln*	*Arg*	*Lys*	Leu	Glu	*Gly*	159–166
Simian OSM†	Val	*Phe*	*Gln*	*Arg*	*Lys*	Leu	Glu	*Gly*	159–166
Human IL-6*	Gln	*Trp*	Leu	Gln	Asp	Met	Thr	Thr	157–164
Mouse IL-6*	Glu	*Trp*	Leu	*Arg*	Thr	Lys	Thr	Ile	157–164
Human IL-11†	Gly	Gly	Ile	*Arg*	Ala	Ala	His	Ala	148–155
Monkey IL-11†	Gly	Gly	Ile	*Arg*	Ala	Ala	His	Ala	148–155
Subgroup IB									
Human G-CSF*	Ala	*Phe*	*Gln*	*Arg*	*Arg*	Ala	Gly	*Gly*	144–151
Mouse G-CSF*	Ala	*Phe*	*Gln*	*Arg*	*Arg*	Ala	Gly	*Gly*	144–151
Chicken MGF†	Pro	*Phe*	*Gln*	Gln	Gln	Val	Gly	*Gly*	147–154
Human GH*	Ser	His	Asn	Asp	Asp	Ala	Leu	Leu	150–157
Human PRL†	Met	Ala	Asp	Glu	Glu	Ser	Arg	Leu	158–165
Human EPO†	Asp	Thr	Phe	*Arg*	*Lys*	Leu	Phe	Arg	136–143
Human IFN α*	Leu	Thr	*Glu*	*Lys*	*Lys*	Tyr	Ser	Pro	131–138
Subgroup II									
Human IL-2†	Glu	*Phe*	Leu	Asn	*Arg*	Trp	Ile	Thr	115–122
Human IL-3*	Glu	*Phe*	Arg	*Arg*	*Lys*	Leu	Thr	Phe	106–113
Human IL-4†	Thr	Leu	*Glu*	Asn	Phe	Leu	Glu	Arg	112–119
Human IL-5†	Gly	Glu	*Glu*	*Arg*	*Arg*	Arg	Val	Asn	106–113
Human GM-CSF†	Thr	*Phe*	*Glu*	Ser	Phe	Lys	Glu	Asn	102–109

Four-helix-bundle proteins are classified into three subgroups: long-chain group proteins that interact with gp130 (IA), long-chain group proteins that do not interact with gp130 (IB), and short-chain group proteins (II) (19, 20). ϕ, Hydrophobic amino acid; Xaa, any amino acid residue; OSM, oncostatin M; PRL, prolactin; EPO, erythropoietin; GM-CSF, granulocyte/macrophage colony-stimulating factor; IFNα, α interferon; MGF, myelomonocytic growth factor.
*Bazan's prediction (11).
†Our prediction.

*Bam*HI–*Pst* I sites of pKKCNTF after being digested with *Bgl* II and *Eco*T22I.

Expression of hCNTF and Its Derivatives. *Escherichia coli* JM109 (*lacI*^{q}) strain, carrying pKK plasmids that have a wild-type or mutant hCNTF gene, was subjected to the induction of protein expression by the addition of isopropyl β-D-thiogalactoside. After the cultivation, cells were lysed with lysozyme, and the supernatant was collected by centrifugation. The aliquots were diluted to phosphate-buffered saline containing 0.1% bovine serum albumin for biological assay. The amount of hCNTF or its mutant proteins in the lysate supernatant was quantified by densitometric scanning on SDS/PAGE after Coomassie brilliant blue staining. The intensity of the bands for mutant proteins was normalized by comparison with purified wild-type hCNTF as a control. Purification of recombinant hCNTF was performed as described by Masiakowski *et al.* (23).

Measurement of Cell Survival Activity. Trophic activities were determined in an assay system of dissociated E10 chicken dorsal root ganglion (DRG) neurons (24). Cells were prepared, dissociated, and plated on a polyornithine–laminin substratum at 4000–5000 neurons per well (96-well microtest plate, Falcon) in Dulbecco's modified Eagle's medium (GIBCO) containing 100 units of penicillin (GIBCO), 100 units of streptomycin (GIBCO), and 250 ng of amphotericin B (GIBCO) per ml; 2 mM L-glutamine; 5% (vol/vol) fetal calf serum (JRH Biosciences, Lenexa, KS); 20 μM 2-deoxyuridine (Nakarai Chemicals, Kyoto); and 20 μM 5-fluorodeoxyuridine (Nakarai Chemical). After incubation for 72 hr, cell survival was measured by MTT (3-[4,5-dimethylthiazol-2-yl]2,5-diphenyltetrazolium bromide) assay. Crude hCNTF in diluted cell lysate showed the same full-dose response curve as did the corresponding purified hCNTF in this biological assay system.

Iodination of hCNTF. Recombinant hCNTF was radiolabeled with Iodo-Gen (Pierce) as described (25). Iodination was done for 5 min at room temperature.

Molecular Modeling of hCNTF Structure. The tertiary structural model of hCNTF was constructed on a Silicon Graphics workstation using QUANTA/CHARMm (Molecular Simulations, Waltham, MA) molecular modeling package with the Protein Design option. The initial structure of hCNTF was generated by using the Cα coordinates of human GH (18) (Protein Data Base 2HHR) as the template. Experimental results were taken into account to determine the secondary structural borders. The initial model was refined by a combination of energy minimization and molecular dynamics at 300 K for 300 psec. The lowest energy structure during the molecular dynamics simulation was selected, and the energy was minimized.

RESULTS

About 180 mutant proteins of hCNTF were created by site-directed mutagenesis by the PCR technique and were analyzed for increased or decreased neural cell survival activity compared with that of wild type. In the AB loop region, point mutations at Met-56 or Gln-63 resulted in a great change of cell survival activity, and in the helix D region, Val-170 or His-174 was shown to be involved in hCNTF recognition to some extent (unpublished data). For example, the mutant protein carrying the Gln-63 → Arg change increased cell survival 5-fold compared with wild-type hCNTF, confirming the previous report of Panayotatos *et al.* (26). Mutant proteins with Val-70 and

His-174 replaced with Ala or Gly and with Leu or Gly, respectively, showed decreased biological activities. Names of mutant proteins reflect the amino acid change—for instance, the protein carrying the Lys-155 → Ala change is called "Lys155Ala" (see Fig. 2).

Biological Activity of hCNTF D1 Cap Region Mutant Proteins. The most striking results were obtained with the mutant proteins with amino acid residue substitutions in the D1 cap region (Fig. 1). The replacement of Lys-155 with any other amino acid residues resulted in complete abolishment or a great decrease of the biological activity (Fig. 2). Methionine, alanine, glycine, threonine, glutamic acid, or tryptophan could not compensate for the lysine residue at all, and the mutant with arginine substitution retained only <1% of the activity of the wild type, implying that not only the positive charge but also the microenvironmental space in the tertiary structure of Lys-155 is strictly recognized by the CNTFR(s). The circular dichroism spectrum of the purified Lys155Ala (and Lys155Arg) mutant hCNTF was similar to that of wild type (unpublished data), indicating that the overall tertiary structure of this mutant protein was well maintained in a similar fashion to that of wild type, although the possibility of some change in structure at the local site(s) could not be excluded. Despite the complete abolishment of cell survival activity, Lys155Ala retained receptor binding ability and competed with the wild type in biological activity, showing an antagonistic feature (Fig. 3). Such an antagonistic phenomenon concomitant with a point mutation has been also observed in other 4-helix-bundle proteins such as IL-2, IL-4, IL-6, granulocyte/macrophage colony-stimulating factor, α interferon, and GH (21, 27–31). Our result indicates that Lys-155 of hCNTF is essential for the receptor binding that functions in signal transduction.

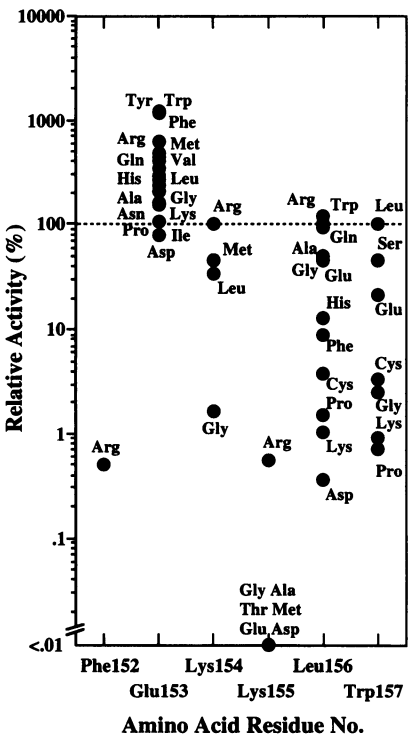

FIG. 1. Relative biological activity of hCNTF mutant proteins with replacements of amino acid residues around the boundary between the CD loop and helix D, which are indicated on the abscissa. The relative activities of hCNTF mutants were determined in an assay system of dissociated E10 chicken DRG neurons based upon full dose–response curves for each mutant compared with that for purified wild-type hCNTF as the control.

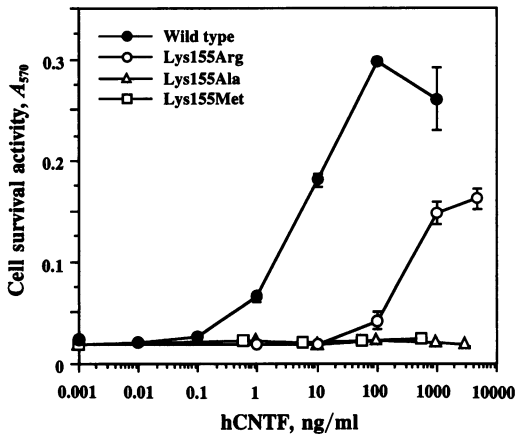

FIG. 2. Neural cell survival activity of hCNTF proteins mutagenized at Lys-155. Dose–response curves of wild-type hCNTF (●) and mutants Lys155Ala (△), Lys155Met (□), and Lys155Arg (○) are shown for cell survival activity measured by an assay system of dissociated E10 chicken DRG neurons.

In addition, many replacements of Glu-153 resulted in an increase of cell survival activity for chicken DRG neurons (Fig. 1) except for the replacement with aspartic acid, which resulted

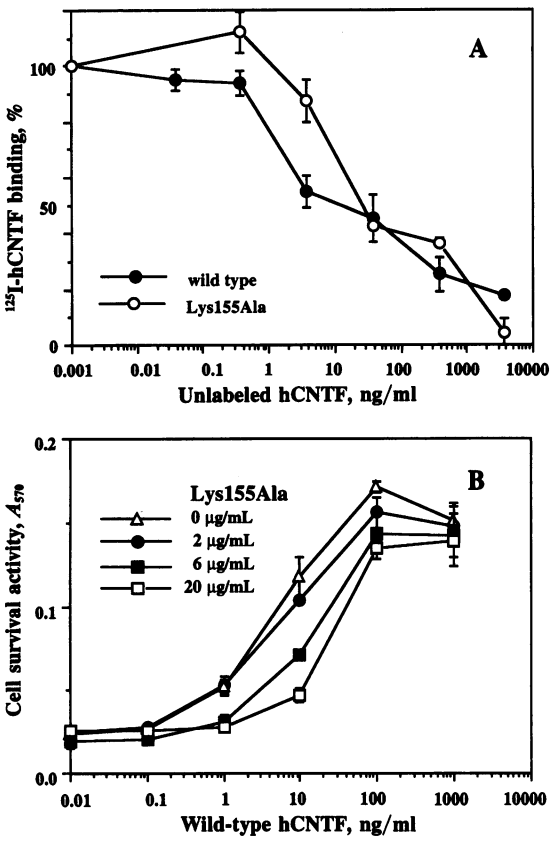

FIG. 3. Antagonistic features of hCNTF proteins mutagenized at Lys-155. (*A*) Competition of nonradioactive wild-type (●) and mutant hCNTF (Lys155Ala) (○) with ^{125}I-radiolabeled wild type (^{125}I-hCNTF) binding to the hCNTFR(s) of human neuroblastoma SH-SY5Y cells. ^{125}I-hCNTF (10 ng/ml) was incubated with human neuroblastoma SH-SY5Y cells (3×10^5/ml) in 0.2 ml in the presence of competing nonradioactive proteins at the indicated concentrations. The ^{125}I-hCNTF radioactivity bound after a 2.5-hr incubation period at 4°C was measured. (*B*) Competitive inhibition of Lys155Ala mutant protein against the neuronal cell survival activity of wild-type hCNTF. Chicken DRG neurons were cultivated with wild type in the absence (△) or presence of 2 (●), 6 (■), and 20 (□) μg of Lys155Ala mutant protein per ml.

in no change of the activity. The replacement of Glu-153 with tyrosine, tryptophan, or phenylalanine resulted in about 5- to 10-fold increased cell survival activity. The increase of activity was also observed for chicken ciliary ganglion neurons and rat DRG neurons (unpublished data), suggesting that this phenomenon is neither species-specific nor neuron-type-specific. The conversions from a negatively charged residue to the corresponding neutral residue (Glu → Gln; Asp → Asn) showed an increase of biological activity, suggesting that some electrostatic feature is important at position 153.

Local Tertiary Structure Around the hCNTF D1 Cap Region and Proposed Interaction of Glu-153 with Lys-155. Since the tertiary structure of hCNTF was not available yet, we constructed a tertiary structure model of hCNTF using as a template the coordinates of human GH, which has the same four antiparallel α-helical structure as CNTF. Fig. 4*A* shows a schematic representation of the structure model with putative

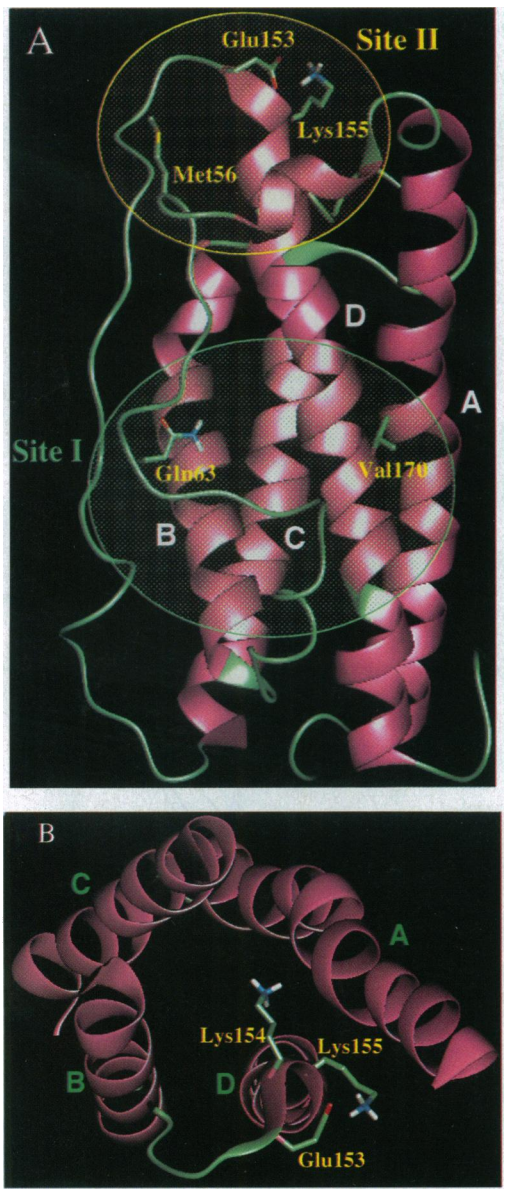

FIG. 4. Predicted tertiary structure model for hCNTF. (*A*) Schematic representation of hCNTF structural model. Predicted helices were helix A (Arg-19 to Leu-44), B (Asn-76 to Val-96), C (His-106 to Leu-130), and D (Glu-153 to Gln-183). Proposed receptor binding sites (sites I and II) are shown as shaded areas. (*B*) Structure of the D1 cap region and the four helices showing the side chains of Glu-153, Lys-154, and Lys-155.

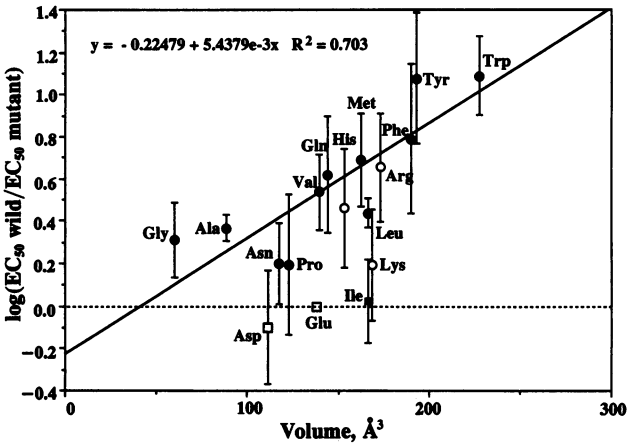

FIG. 5. Correlation of the volume of the amino acid residue mass at position 153 with the cell survival activity of chicken DRG. The ratio of a median effective concentration of wild-type hCNTF (EC_{50} wild) to that of the respective mutant hCNTF (EC_{50} mutant) is expressed by the logarithmic value on the vertical axis. Neutral residues (●) except for isoleucine (■), positively charged (○), and negatively charged (□) residues are expressed. The dots represent three to eight independent experiments, and the bars indicate the SD.

receptor binding sites. The boundary between the CD loop and helix D was determined by evaluating the activities of mutant proteins in which an amino acid residue had been replaced by a proline residue, a strong helix breaker. Glu153Pro retained biological activity, whereas neither Leu156Pro nor Trp157Pro did. These demonstrated that at least Leu-156 and Trp-157 belonged to helix D, and Glu-153 was likely to locate at the boundary.

Our structural model of hCNTF predicted that Glu-153 and Lys-155 would face a receptor component of hCNTF in the same direction, whereas Lys-154 would face in the opposite direction (Fig. 4*B*) and that Glu-153 in wild-type hCNTF interacts through hydrogen bonding with Lys-155, a residue strictly recognized by the receptor electrostatically and spatially as described above. Thus, the Glu-153 residue in wild type might interfere with the binding of Lys-155 to the receptor(s). The hydrogen bonding between these two residues remained intact during most of the molecular dynamics simulation at 300 K for 300 psec with water molecules surrounding the D1 cap region (unpublished data). The replacement of Glu-153 with a neutral or a positively charged residue might diminish the intramolecular electrostatic interaction with Lys-155 and consequently increase the receptor binding through Lys-155. Alternatively, the direct interaction of Glu-153 with receptor(s) also should be taken into consideration as an another explanation for the increased activity of proteins mutated at Glu-153. The cell survival activity of mutant proteins correlated well with the mass volume of respective amino acid residues at position 153 (Fig. 5). In the case of neutral residues except isoleucine, the larger mass volume gave higher biological activity with a correlation coefficient of 0.703. Either mutant Glu153Tyr or Glu153Trp showed an activity about 5- to 10-fold higher than that of wild type.

DISCUSSION

Here we propose that hCNTF has at least two receptor binding sites (sites I and II shown in Fig. 4*A*) involved in its biological activity. Site I consists of both helix D and the AB loop in which Gln-63 plays a key role. Site II corresponds mainly to the D1 cap region, especially the D1 motif, including Glu-153 and Lys-155. Site I has already been proposed to be involved in receptor recognition in hCNTF (26) as well as in other α-helical cytokines (15–18). The results presented here suggest

that site II is also involved in receptor recognition, signal transduction, and cell survival activity of hCNTF. The involvement of the boundary region of the CD loop and helix D in receptor recognition has also been suggested for IL-6 (21) and LIF (22), although the D1 motif involved in receptor recognition has not been characterized yet. The D1 cap region might be important in receptor recognition for not only CNTF, IL-6, and LIF but also oncostatin M and IL-11—in other words, all of the gp130-reactive and long-chain group four-helix-bundle proteins. Among these proteins, LIFR is also common to LIF, oncostatin M, and CNTF. LIF, oncostatin M, and IL-11 generate intracellular signals through ligand-mediated heterodimerization of a ligand-specific receptor subunit and gp130 (32). IL-6 transduces signaling via gp130 homodimerization, which is arbitrated by the complex of IL-6 with its receptor (IL-6R) (32). CNTF acts by heterodimerization of gp130 with LIFR following the formation of the CNTF–CNTFR complex (12–14). What is the function of the D1 cap region, and especially of the D1 motif in the region, for receptor recognition and signal transduction in these proteins? To which component(s) of the receptor would the D1 cap region and the D1 motif in the region directly interact? These questions remain to be answered. The D1 cap region (site II) of IL-6 has been shown to be involved in high-affinity receptor binding (21). Our results predict that the D1 motif in site II of hCNTF is involved in that. Receptor molecule(s) involved in high-affinity receptor binding are not the ligand-specificity-determining receptor subunit but are the transducing homodimer of gp130 or the heterodimer of gp130 and LIFR. The D1 motif is not conserved in all gp130-responsive proteins. The D1 motif of IL-6 and IL-11 has a low sequence similarity to the D1 motif consensus sequence (Table 1). In contrast, the D1 motif of CNTF, LIF and oncostatin M to which LIFR is common as a receptor component possesses an extremely high homology to the consensus sequence, and hCNTF Lys-155 corresponds to human LIF Lys-158 and human oncostatin M Lys-163, respectively (Table 1). Hence, it is likely that Lys-155 of the hCNTF D1 motif in the D1 cap region might be strictly recognized by LIFR and that the D1 cap region sequence other than the D1 motif might be directly or indirectly involved in the interaction with the common receptor component gp130 in some ways. Meanwhile, G-CSF has a high similarity to the consensus sequence in the D1 motif (Table 1) and might utilize this D1 cap region for receptor recognition, although there is no evidence that G-CSF interacts with gp130 or LIFR. This expectation was also supported by the fact that a monoclonal antibody against the boundary region between the CD loop and helix D of G-CSF neutralized its activity (33).

A good correlation of the cell survival activity of mutant proteins with the mass volume of the amino acid at position 153 suggests that there might be a large cavity in the receptor(s) to which residue 153 of hCNTF interacts. An amino acid residue with a large mass volume might get rid of the water molecules from the cavity and increase the hydrophobic or electrostatic interaction between residue 153 of hCNTF and its receptor(s).

Herein, we show that the Lys-155 residue is essential for the biological activity of hCNTF and that the D1 cap region is functionally important as one of the putative receptor recognition sites. The increased biological activity of the hCNTF mutagenized at Glu-153 suggests that these mutants could be good candidates for a second-generation hCNTF. Recently, clinical trial with a recombinant hCNTF has disclosed that a relatively high dosage of hCNTF might cause side effects. A second-generation hCNTF with a higher activity might help to overcome the drawback. The structure–function relationship described here may be useful in developing a new CNTF derivative for therapy of neurodegenerative diseases singly or in combination with brain-derived neurotrophic factor (34, 35).

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