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Identification of ciliary neurotrophic factor (CNTF) residues essential for leukemia inhibitory factor receptor binding and generation of CNTF receptor antagonists

(ciliary neurotrophic factor/leukemia inhibitory factor/gp130)

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ABSTRACT **Ciliary neurotrophic factor (CNTF) drives the sequential assembly of a receptor complex containing the ligand-specific α-receptor subunit (CNTFRα) and the signal transducers gp130 and leukemia inhibitory factor receptor-β (LIFR). The D1 structural motif, located at the beginning of the D-helix of human CNTF, contains two amino acid residues, F152 and K155, which are conserved among all cytokines that signal through LIFR. The functional importance of these residues was assessed by alanine mutagenesis. Substitution of either F152 or K155 with alanine was found to specifically inhibit cytokine interaction with LIFR without affecting binding to CNTFRα or gp130. The resulting variants behaved as partial agonists with varying degrees of residual bioactivity in different cell-based assays. Simultaneous alanine substitution of both F152 and K155 totally abolished biological activity. Combining these mutations with amino acid substitutions in the D-helix, which enhance binding affinity for the CNTFRα, gave rise to a potent competitive CNTF receptor antagonist. This protein constitutes a new tool for studies of CNTF function in normal physiology and disease.**

Ciliary neurotrophic factor (CNTF) is a protein expressed by Schwann cells and astrocytes, which exerts potent stimulatory effects on the survival and differentiation of a variety of neuronal and glial cells and has been proposed to act as a lesion factor involved in the prevention of neuronal degeneration following injury (1, 2). Studies of the physiological or pathophysiological roles of CNTF would be facilitated by the availability of specific, high-affinity receptor antagonists. Like other growth factors and cytokines controlling essential cell functions such as survival, proliferation, and differentiation, CNTF exerts its actions through the binding, sequential assembly, and activation of a multi-subunit receptor complex (3, 4). The identification of the protein domains that participate in interactions with different receptor subunits is a prerequisite for understanding the mechanism of receptor activation and for the design of specific antagonists (5–8).

CNTF belongs to a group of functionally related proteins of the long-chain α-helical cytokine superfamily, including interleukin-6 (IL-6), leukemia inhibitory factor (LIF), oncostatin M, IL-11, and cardiotrophin-1 (CT-1) (4, 9–12), which signal through structurally related and partially shared receptor subunits. The CNTF receptor complex is composed of a low-affinity, ligand-specific α-receptor (CNTFRα), which is predominantly expressed in neuronal cells (13), and two more widely distributed signal-transducing subunits, namely gp130 and the LIF receptor-β (LIFR). Binding of CNTF to CNTFRα triggers the subsequent association of gp130 and LIFR in a high-affinity receptor complex (3). By analogy to the hexameric receptor complex assembled by IL-6 (14), the CNTF receptor is thought to contain two cytokine molecules, two α-receptor and two signal-transducing subunits (15). Heterodimerization of the latter leads to the activation of a signal transduction cascade mediated by cytoplasmic tyrosine kinases of the JAK and Src families (3, 4). The related cytokines of this family all use gp130 as a signal transducer and affinity converter. Signaling occurs through homodimerization of gp130 (for IL-6) or heterodimerization of gp130 and LIFR (for LIF and CT-1) (4, 16).

Binding sites for CNTFRα and gp130 on the surface of CNTF (17–20) are thought to be located at positions analogous to receptor binding sites 1 and 2 of IL-6 (6). The binding site for LIFR has not been identified as yet. Interaction of LIF with LIFR was shown (8, 21) to involve a region equivalent to site 3 of IL-6 (7, 14, 22), located at the top of helix D within the D1 structural motif (10). Recent mutational studies suggested that this region is also important for CNTF-receptor activation (23). In this paper, we describe the functional role of specific D1 residues in LIFR binding and the development of a potent competitive CNTF-receptor antagonist.

MATERIALS AND METHODS

Generation of CNTF Mutants. Mutations were introduced into the human CNTF or S166D/Q167H/CNTF (DH-CNTF) sequences by inverse PCR (24), using the pRSET-CNTF and pRSET-MUT-DH vectors (18) as templates. Protein expression and isolation from bacterial inclusion bodies were performed as described (18). Additional purification was carried out by HPLC using a preparative C4 column (Vydac 214TP, 2.2 × 25 cm, 10 μm) eluted at a flow rate of 30 ml/min with a linear gradient of 40–60% acetonitrile/0.1% trifluroacetic acid in water/0.1% trifluroacetic acid. *n*-Octylglucopyranoside [0.1% (wt/vol)] was added to the eluates prior to removal of the solvent by lyophilization. Proteins were resuspended in water and stored at 4°C.

CNTFRα-Binding Assay. The ability of CNTF variants to compete with biotinylated CNTF (8 nM) for binding to the extracellular domain of myc-tagged CNTFRα was determined in a solid-phase binding assay, in the presence of soluble (s)-gp130, as described (18). In some experiments, biotinylated DH-CNTF (0.5 nM), prepared according to Saggio *et al.* (25),

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Abbreviations: CT, cardiotrophin; Chat, choline acetyltransferase; CNTF, ciliary neurotrophic factor; CNTFRα, CNTF α-receptor; IL, interleukin; LIF, leukemia inhibitory factor; LIFR, LIF receptor; s-, soluble; DH-CNTF, S166D/Q167H/CNTF; FA-CNTF, K155A/CNTF; AA-CNTF, F152A/K155A/CNTF; AKDH-CNTF, F152A/S166D/Q167H/CNTF; FADH-CNTF, K155A/S166D/Q167H/CNTF; AADH-CNTF, F152A/K155A/S166D/Q167H/CNTF.

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was used as the ligand. Consistent with its increased affinity for CNTFRα, inhibition of this ligand required higher concentrations of competitors (IC_{50} of CNTF: 113 ± 20 nM with biotinylated DH-CNTF vs. 14 ± 2 nM with biotinylated CNTF, mean ± SEM; $n = 3$). However, the relative binding activity of all tested CNTF variants (IC_{50} of wild type/IC_{50} of variant) did not depend on the choice of biotinylated ligand.

Assembly of Receptor Complexes *in Vitro*. The binding of ^{35}S-labeled s-gp130 and s-LIFR to cytokine/CNTFRα complexes was determined by immunoprecipitation experiments as described (15).

Bioassays. Stimulation of TF-1 cell proliferation and haptoglobin secretion from HepG2 cells was determined as described (18), except that the HepG2 assay was performed in 96-well culture plates. The effects of CNTF variants on choline acetyltransferase (Chat) activity in IMR-32 cells were determined as reported (26). For determinations of Chat induction in the murine septal neuron × neuroblastoma hybrid cell line SN-56 (27), cells were plated at a density of 40,000 cells per well in 24-well culture plates and incubated for 3 days with or without CNTF variants in 0.5 ml of culture medium (DMEM containing 10% fetal bovine serum and 50 μg/ml gentamicin). Chat activity was then determined in aliquots of cell extracts as described (26).

RESULTS

Identification of a LIFR-Binding Site on Human CNTF. Alignment of the CNTF sequences from human, rabbit, and rat with those of the CNTF-related chicken growth promoting activity (28) and other LIFR-binding cytokines such as LIF, oncostatin M, and CT-1 reveals the presence of two conserved amino residues within the D1 structural motif (10) (Table 1). To determine the functional importance of human CNTF residues F152 and K155, we generated variants in which these residues were replaced by alanine. The effect of the mutations was also examined in the background of the DH-CNTF variant (S166D/Q167H), which was previously shown to possess enhanced CNTFRα-binding affinity (18).

Alanine substitution of F152 and K155 did not modify the interaction with CNTFRα, as determined in a competition binding assay. Thus, K155A/CNTF (FA-CNTF) and F152A/K155A/CNTF (AA-CNTF) were equipotent with wild-type CNTF in displacing the binding of biotinylated CNTF or biotinylated DH-CNTF to immobilized CNTFRα (Table 2). As reported (18), DH-CNTF was ≈40-fold more potent than CNTF in this assay. The variants F152A/S166D/Q167H/CNTF (AKDH-CNTF), K155A/S166D/Q167H/CNTF (FADH-CNTF), and F152A/K155A/S166D/Q167H/CNTF (AADH-CNTF) were equipotent with DH-CNTF in binding to CNTFRα (Table 2). These results show that F152 and K155 do not participate in binding to the CNTFRα, in agreement with the view that the CNTFRα binding site (site 1) is located in the C-terminal portion of the D-helix (9, 18–20).

We previously demonstrated that s-gp130 and s-LIFR are able to bind independently to the CNTF/CNTFRα subcomplex (15). To assess the functional importance of D1 motif residues, the ability of CNTF variants to assemble complexes with the different signal-transducing receptor subunits was determined. As shown in Fig. 1*A*, alanine substitution of F152 or K155 did not affect the binding of the DH-CNTF/CNTFRα complex to s-gp130. In contrast, variants bearing these mutations possessed no detectable binding to s-LIFR (Fig. 1*B*). Accordingly, these variants were unable to assemble the tripartite CNTFRα/LIFR/gp130 complex, which is thought to correspond to the physiologically active form of CNTF receptor assembled on the cell surface (Fig. 1*C*). These results

Table 1. D1 motif sequences of CNTF-related cytokines

Cytokine	Sequence
Human CNTF	GL**F**EK**K**LWG
Rat CNTF	GL**F**EK**K**LMG
Rabbit CNTF	GL**F**EK**K**LWG
Chicken GPA	SL**F**EQ**K**LRG
Human LIF	DV**F**QK**K**KLG
Mouse LIF	EA**F**QR**K**KLG
Human OM	DA**F**QR**K**LEG
Mouse CT-1	GI**F**SA**K**VLG
Human IL-6	NQWLQDMTT
Human IL-11	SAWGGIRAA

Alignments are from refs. 10–12, 21, and 28. GPA, growth promoting activity; OM, oncostatin M.

Table 2. CNTFRα-binding activity of CNTF variants

Protein	Mutations	Relative binding, mean ± SEM
CNTF	—	1.0
DH-CNTF	S166D/Q167H	41 ± 4
FA-CNTF	K155A	1.0 ± 0.1
FADH-CNTF	K155A/S166D/Q167H	42 ± 2
AKDH-CNTF	F152A/S166D/Q167H	32 ± 11
AA-CNTF	F152A/K155A	0.9 ± 0.1
AADH-CNTF	F152A/K155A/S166D/Q167H	51 ± 19

Relative binding is the ratio (IC_{50} of CNTF)/(IC_{50} of tested protein). Data are mean values from 2–4 experiments.

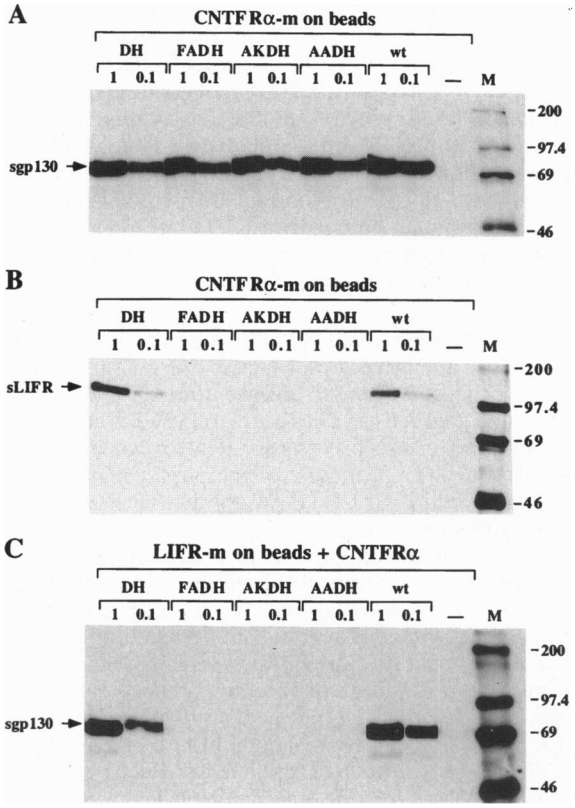

FIG. 1. Binding of CNTF variants to signal-transducing receptor subunits. (*A*) Assembly of the CNTFRα/gp130 complex. Myc-tagged s-CNTFRα was immobilized on protein A-Sepharose beads via an anti-myc antibody and incubated with ^{35}S-labeled s-gp130 in the absence (−) or in the presence of different amounts (0.1 or 1 μg) of CNTF variants, as indicated on top of the lanes. After washing the beads, bound material was eluted and subjected to SDS/PAGE followed by autoradiography (15). (*B*) Assembly of the CNTFRα/LIFR complex. Immobilized CNTFRα was incubated with ^{35}S-labeled s-LIFR in the presence of 0.1 or 1 μg of the indicated CNTF variants. (*C*) Assembly of the CNTFRα/gp130/LIFR complex. Immobilized myc-tagged LIFR was incubated with s-CNTFRα, ^{35}S-labeled s-gp130, and 0.1 or 1 μg of the indicated CNTF variants. wt, CNTF; m, myc; M, molecular weight markers expressed in kDa.

indicate that F152 and K155 of CNTF are part of a LIFR-binding site that is distinct from the site of interaction with gp130.

Biological Activity of CNTF Mutants Impaired in LIFR Binding. CNTF variants with impaired LIFR binding are expected to possess reduced biological activity. The human hepatoma cell line HepG2 does not express CNTFRα and responds only to high concentrations of the cytokine, probably because of a low-affinity interaction of CNTF with cellular LIF receptors (18, 29). As previously reported (18), CNTF and DH-CNTF were equipotent in stimulating haptoglobin production in HepG2 cells, with EC_{50} values of ≈100 ng/ml (Fig. 2*A*). In agreement with the view that this effect is mediated by LIFR, amino acid substitutions that impair LIFR binding led to a strong reduction in biological activity. Thus, variants bearing alanine substitutions of F152 or K155 produced only modest effects (≈20% of maximal) at high concentrations (10 μg/ml). Simultaneous mutation of both residues totally abolished activity (Fig. 2*A*).

In the presence of exogenous s-CNTFRα, cells bearing LIFR and gp130 become sensitive to low concentrations of CNTF, due to formation of the high-affinity tripartite receptor complex (3). As described (18), addition of s-CNTFRα (from 8 to 800 ng/ml) led to a shift of the CNTF dose-response curve toward lower concentrations (Fig. 2 *B–D*). A minimal EC_{50} was eventually obtained, which reflects saturation of the ligand at receptor concentrations higher than the ligand/receptor equilibrium dissociation constant (30) (Fig. 2*D*). Because DH-CNTF has a 40-fold higher affinity for CNTFRα than CNTF, saturation of this variant was reached at lower receptor concentrations than for the wild-type cytokine (Fig. 2 *C* and *D*). Alanine substitution of K155 greatly diminished, but did not totally abolish biological activity. Thus, the activity of FA-CNTF was barely detectable at a receptor concentration of 8 ng/ml (Fig. 2*B*), but could be augmented by increasing the concentration of s-CNTFRα (Fig. 2 *C* and *D*). Consistent with its higher affinity for CNTFRα, FADH-CNTF exhibited significantly higher potency than FA-CNTF when compared at subsaturating receptor concentrations, and reached its minimal EC_{50} at lower receptor concentrations (Fig. 2 *B* and *C*). At a saturating concentration of CNTFRα (800 ng/ml), both K155A variants (FA-CNTF and FADH-CNTF) behaved as weak partial agonists, with 2.5-fold lower maximal responses and at least 100-fold higher EC_{50} values than CNTF (Fig. 2*D*).

Alanine substitution of F152A had a less drastic effect on potency. At saturating concentrations of s-CNTFRα, the maxi-

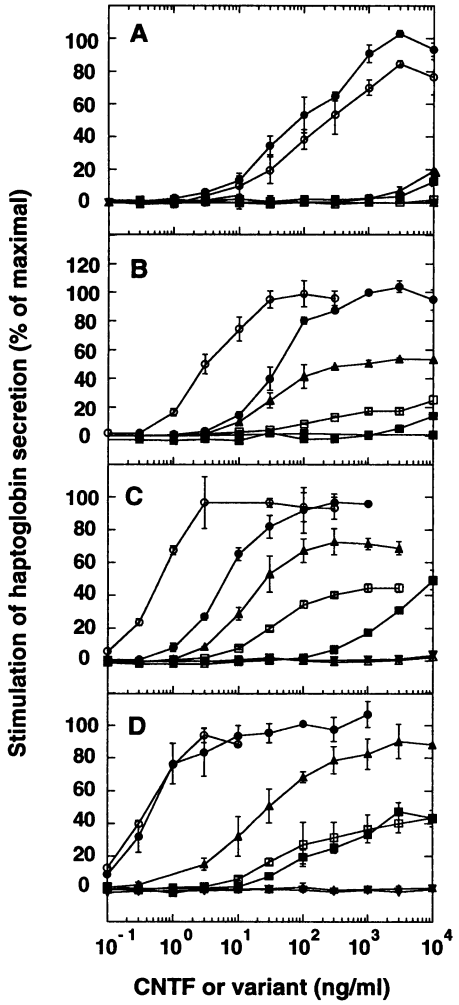

FIG. 2. Stimulation of haptoglobin production in HepG2 cells by CNTF and CNTF variants. Experiments were performed either in the absence (*A*) or presence of s-CNTFRα at the following concentrations: 8 ng/ml (*B*), 80 ng/ml (*C*), 800 ng/ml (*D*). The proteins tested were CNTF (●), DH-CNTF (○), FA-CNTF (■), FADH-CNTF (□), AKDH-CNTF (▲), AA-CNTF (△), and AADH-CNTF (▼). Each point is the mean ± SEM from 2–4 separate experiments. Data are expressed as a percentage of the maximal CNTF effect to normalize for differences in absolute responses between different experiments. For typical stimulation ratios see Fig. 4.

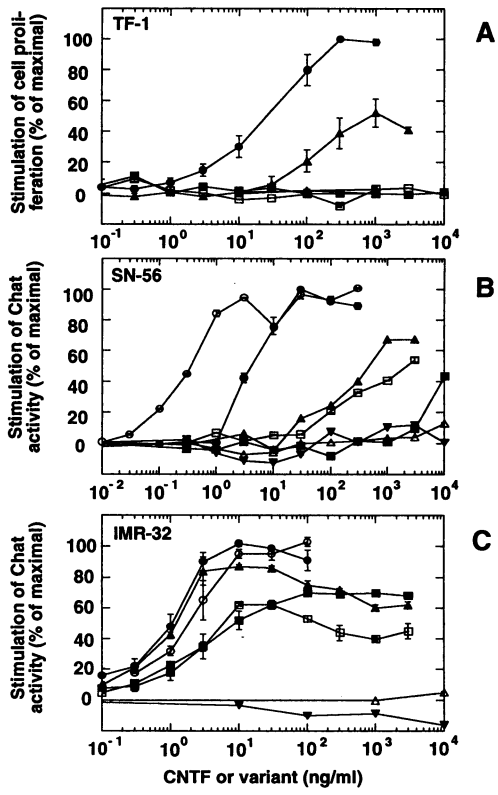

FIG. 3. Biological effects of CNTF variants in human and murine cell lines. (*A*) Stimulation of TF-1 cell survival. Experiments were performed in the presence of 80 ng/ml of s-CNTFRα. (*B*) Stimulation of Chat activity in SN-56 cells. (*C*) Stimulation of Chat activity in IMR-32 cells. The proteins tested were CNTF (●), DH-CNTF (○), FA-CNTF (■), FADH-CNTF (□), AKDH-CNTF (▲), AA-CNTF (△), and AADH-CNTF (▼). Each point is the mean ± SEM from 2–4 separate experiments (*A* and *C*) or from a single experiment performed in duplicate culture dishes (*B*). Data are expressed as a percentage of the maximal CNTF effect to normalize for differences in absolute responses between different experiments. At supersaturating concentrations, CNTF led to an ≈5-fold increase in TF-1 cell numbers (18) and a 3- to 4-fold increase in Chat activity in SN-56 and IMR-32 cells (see Fig. 5*A*).

mal effect elicited by AKDH-CNTF was 60–80% that of DH-CNTF, with an ≈20-fold increased EC_{50}. However, simultaneous mutation of F152A and K155A, either in the context of CNTF or of DH-CNTF, led to a complete loss of potency, with no detectable signal even at the highest concentrations of receptor (800 ng/ml) and variant (10 μg/ml) tested (Fig. 2*D*).

The activities of CNTF variants were also tested in the TF-1 cell survival assay (18, 31) in the presence of 80 ng/ml of s-CNTFRα. In this system, FA-CNTF, FADH-CNTF, AA-CNTF, and AADH-CNTF were totally inactive at concentrations up to 10 μg/ml. The variant AKDH-CNTF acted as a partial agonist, with a maximal effect of ≈50% of that elicited by DH-CNTF, and an ≈100-fold higher EC_{50} (Fig. 3*A*).

The effects of the variants on membrane-bound CNTF receptor were tested in cells of neuronal origin. CNTF enhances the cholinergic phenotype of the murine SN-56 cell line, which has been shown to maintain morphological and functional characteristics of differentiated septal neurons (27, 32). As shown in Fig. 3*B*, the potency of CNTF to stimulate Chat expression in SN-56 cells was strongly reduced by mutations in the LIFR-binding site. Thus, weak activity of FA-CNTF was observed only at a concentration of 10 μg/ml. FADH-CNTF and AKDH-CNTF were equipotent and ≈100- and 1000-fold less active than CNTF and DH-CNTF, respectively. Simultaneous mutation of F152 and K155, in either AA-CNTF or AADH-CNTF, led to a complete loss of biological activity (Fig. 3*B*).

We next tested the effects of CNTF variants on Chat expression in the human neuroblastoma cell line IMR-32 (26). In contrast to all other cells tested, CNTF and DH-CNTF were equipotent in this system (EC_{50} ~ 1 ng/ml), probably due to a high local receptor concentration, leading to virtually unidirectional capture (33) of either ligand (Fig. 3*C*). That the CNTF receptor was saturating with respect to ligand is supported by the finding that the effects of CNTF or DH-CNTF were not potentiated by addition of s-CNTFRα (data not shown). IMR-32 cells were also less sensitive than the other tested cells to the effect of single mutations in the LIFR-binding site of CNTF. Thus, AKDH-CNTF was as active as the wild-type cytokine, and even the K155A variants FA-CNTF and FADH-CNTF retained considerable agonistic potency. Yet, simultaneous mutation of both F152 and K155 produced a strong synergistic inhibition of biological activity. Indeed, AA-CNTF and AADH-CNTF were totally inactive up to concentrations of 10 μg/ml (Fig. 3*C*).

Antagonistic Properties of CNTF Mutants Impaired in LIFR Binding. CNTF analogs that are unable to interact with LIFR, but have retained the ability to recruit CNTFRα, are expected to behave as competitive antagonists. Inhibition of CNTF or DH-CNTF action in HepG2 cells was tested in the presence of 80 ng/ml or 8 ng/ml of s-CNTFRα, respectively. AADH-CNTF caused a dose-dependent, parallel shift of the CNTF (Fig. 4*A*) and DH-CNTF (Fig. 4*B*) dose-response curves toward higher concentrations. Because AADH-CNTF does not interact with LIFR, it should not inhibit the CNTFRα-independent activity of CNTF in HepG2 cells. Indeed, the antagonist, in the presence of CNTFRα, shifted the EC_{50} for CNTF up to a limiting value (≈100 ng/ml), which precisely corresponds to that observed in the absence of α-receptor (Fig. 4*A*). Likewise, the residual activity of DH-CNTF in the presence of a high concentration of AADH-CNTF (10 μg/ml) was of the same magnitude as its CNTFRα-independent effect (Fig. 4*B*). These results show that AADH-CNTF is a competitive antagonist of the CNTFRα, but not of other CNTF-responsive cytokine receptors (presumably LIFR) present in HepG2 cells. The specificity of AADH-CNTF was confirmed by its complete failure (at concentrations up to 10 μg/ml) to inhibit LIF-induced or IL-6-induced haptoglobin production in HepG2 cells (data not shown).

The ability of CNTF variants to competitively inhibit CNTF action should be correlated with their affinity for the α-receptor. Half-maximal inhibition of CNTF (Fig. 4*C*) or DH-CNTF (Fig. 4*D*) activity on s-CNTFRα in HepG2 cells was obtained with 30- to 100-fold molar excess of AADH-CNTF over agonist. In contrast, the weaker CNTFRα binder AA-CNTF did not significantly inhibit agonist action, even when used at 1,000- to 10,000-fold molar excess (Fig. 4 *C* and *D*).

The antagonistic activity of AADH-CNTF was also tested on receptor complexes containing membrane-bound

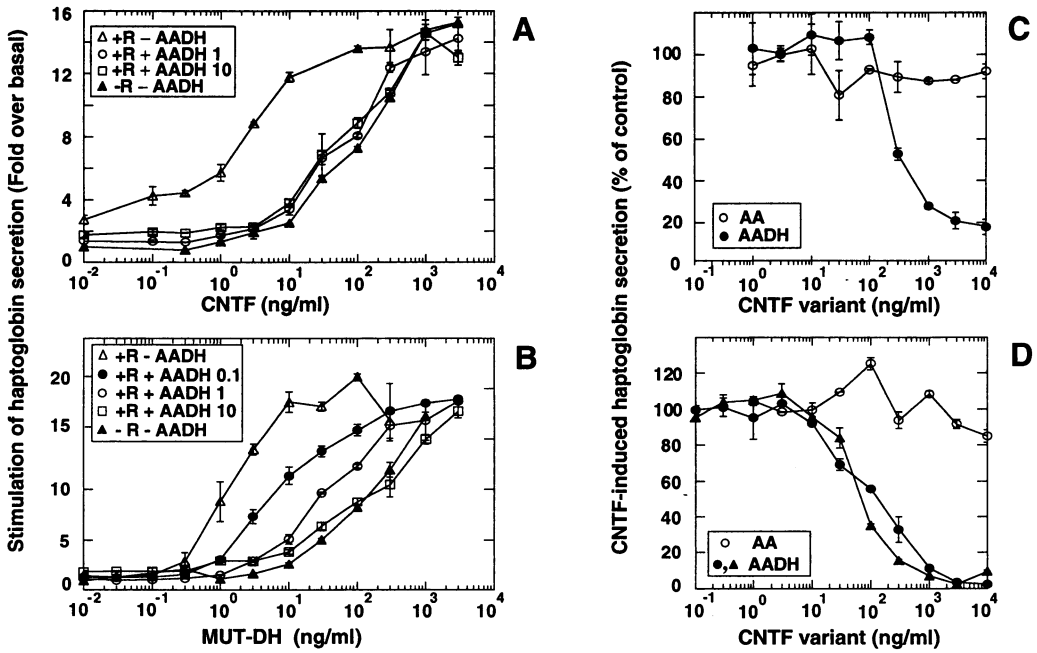

FIG. 4. Antagonistic properties of AADH-CNTF in HepG2 cells. The effects of CNTF (*A*) and DH-CNTF (*B*) on haptoglobin production were determined in the absence (−R) or presence (+R) of 8 ng/ml CNTFRα and AADH-CNTF at the indicated concentrations (in μg/ml). (*C*) Effect of increasing concentrations of AA-CNTF and AADH-CNTF on the response induced by 10 ng/ml of CNTF in the presence of 80 ng/ml CNTFRα. (*D*) Effect of increasing concentrations of AA-CNTF and AADH-CNTF on the response induced by 1 ng/ml (▲) or 3 ng/ml (●) of DH-CNTF in the presence of 8 ng/ml CNTFRα.

CNTFRα. As shown in Fig. 5*A*, the protein caused a dose-dependent right shift of the CNTF dose-response curve in IMR-32 cells. Half-maximal inhibition of CNTF-induced Chat activity was obtained with an ≈70-fold excess of AADH-CNTF (IC_{50} = 200 ng/ml or 8 nM; Fig. 5*B*). In this system, the effect of CNTF was also inhibited by AA-CNTF. However, the ≈40-fold lower CNTFRα-binding affinity of this protein, relative to that bearing the DH mutations, was reflected by a 40-fold higher IC_{50} (8 μg/ml or 300 nM; Fig. 5*B*). Finally, AADH-CNTF fully antagonized the effect of CNTF (10 ng/ml) also in SN-56 cells with an IC_{50} of 1 ± 0.2 μg/ml (42 ± 8 nM; n = 2), corresponding to 100-fold molar excess.

DISCUSSION

Functional CNTF receptor activation requires heterodimerization of the signal-transducing subunits within the tripartite CNTFRα/gp130/LIFR receptor complex (3). CNTF variants that interact with the CNTFRα, but not with either gp130 or LIFR, are therefore expected to be biologically inactive while retaining the ability to compete with the wild-type protein for receptor binding. In the present work, the design of a competitive neurotrophic factor receptor antagonist was rendered possible by the identification of a specific LIFR-binding site on the surface of CNTF.

CNTF is expected to possess at least three receptor binding sites for interaction with the ligand-specific α-receptor and the signal-transducing receptor subunits gp130 and LIFR. Binding sites for CNTFRα (site 1) and gp130 (site 2) have been proposed to include residues in the C-terminal part of helix D and the A/B loop, and in helix A, respectively (17–20). By analogy to IL-6 and LIF (7, 8, 14, 21, 22), site 3 might include the D1 motif at the beginning of helix D, which in human CNTF is part of an accessible surface located on top of the cytokine molecule (9). In agreement with this notion, amino acid substitutions within this region were recently shown to strongly affect the biological activity of CNTF on cultured chicken neurons. In particular, FA-CNTF was reported to be completely inactive in this system and to weakly antagonize the effect of CNTF (23). Even though these results suggested that the variant was impaired in its interaction with a signal-transducing receptor subunit, this was not directly tested. The present finding that amino acid substitutions in the D1 motif inhibit interaction only with LIFR but not with gp130 argues for the existence of distinct binding sites for each signal transducer, as represented schematically in Fig. 6. This situation is in keeping with the asymmetry of the CNTF receptor complex (15) and contrasts with the symmetrical nature of the IL-6 receptor complex, characterized by homodimerization of gp130 (14). The LIFR specificity of site 3 is consonant with the strong conservation of the critical F and K residues among all cytokines (growth promoting activity, LIF, oncostatin M, CT-1) currently known to use LIFR for signaling (8, 12, 16, 21, 28). In contrast, these amino acids are not present in IL-6 and IL-11, which interact with gp130, but not with LIFR (4, 11) (see Table 1). A specific function of these residues is also suggested by the finding that alanine substitution of the adjacent K154 residue of human CNTF did not affect biological activity (unpublished results).

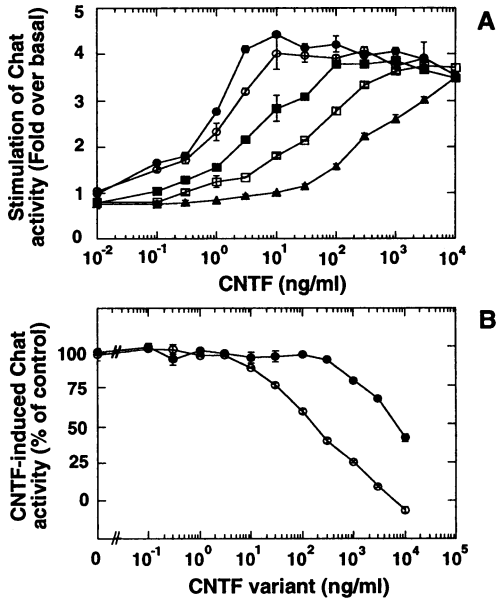

FIG. 5. Antagonistic properties of F152A/K155A variants in IMR-32 cells. (*A*) CNTF-induced Chat activity in the absence (●) or presence of the following concentrations of AADH-CNTF (in μg/ml): 0.01 (○), 0.1 (■), 1 (□), 10 (▲). (*B*) Effects of increasing concentrations of AA-CNTF (●) and AADH-CNTF (○) on the response induced by 3 ng/ml of CNTF.

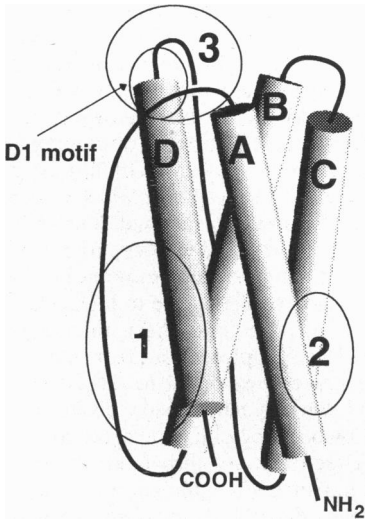

FIG. 6. Schematic model of the sites of interaction of CNTF with its receptor subunits.

Whether F152 and K155 participate directly in LIFR binding or whether they are essential for the correct folding of the LIFR epitope remains to be determined by structural analysis of the CNTF receptor complex. Further mutational studies will also help to assess whether the D1 motif constitutes the major LIFR determinant, as proposed for LIF (8), or whether this binding site is more extended, as in the case of the composite site 3 of IL-6, which was shown to include residues from the AB loop (22). In any case, the present results, together with previous structure–function studies of CNTF (18–20), are in line with a growing body of evidence indicating that neuropoietic cytokines use topologically conserved epitopes for receptor binding (ref. 22 and references therein).

In direct binding experiments, formation of LIFR-containing receptor complexes could not be detected with either the F152A or the K155A mutants, consistent with their inability to functionally activate the LIF receptor in human hepatoma cells. Yet, in the presence of CNTFRα, neither of these mutations invariably inhibited signaling. These results indicate that both mutants retained the ability to assemble low levels of tripartite receptor, undetectable by direct binding, but sufficient to trigger biological effects in cells with high signal amplification. The dependence of biological potency on the receptor concentration (ref. 30; Fig. 2) explains why mutations that reduce the LIFR-binding affinity of CNTF gave rise to partial agonists with greatly varying activities on different cells. For instance, FA-CNTF, which was reported to be totally inactive in a chicken neuronal survival assay (23), possessed

bioactivities on human cell lines ranging from very low (TF-1) through intermediate (HepG2) to high (IMR-32).

The F152A and K155A variants constitute a new class of CNTFRα-selective agonists, which are expected to display a more restricted pharmacological profile than the wild-type cytokine. For instance, the reduced bioactivity of these proteins in human hepatoma cells suggests that they should be relatively poor inducers of the acute-phase response, which is mediated by LIFR bearing hepatocytes, possibly via circulating s-CNTFRα (2). On the other hand, the F152A and K155A variants potently induced cholinergic differentiation of human neuroblastoma cells. High potency in this system could be due to high cell surface concentrations (33) of LIFR (leading to near unidirectional capture of cytokine/CNTFRα complexes) and/or membrane-anchored CNTFRα. The latter is expected to diffuse more slowly than s-CNTFRα, and this may lead to slow dissociation and efficient local reassociation of the cytokine/receptor complex. Such mechanisms would effectively counterbalance the effect of cytokine mutations that impair LIFR interaction. It will be interesting to determine whether the F152A and K155A variants retain high potency on other cells expressing high levels of CNTFRα and/or LIFR, such as neurons participating in motor functions (13, 34). Analogs that act on CNTF-responsive neurons but elicit reduced peripheral side effects (such as acute-phase response) may possess increased therapeutic potential.

In contrast to the individual alanine substitution of either F152 or K155, their combined mutation abolished biological activity in all tested bioassays, consistent with total inhibition of LIFR interaction and signaling. Even though the double-mutant AA-CNTF was equipotent with wild type in binding to CNTFRα and gp130, it antagonized the biological effects of CNTF only at high concentrations, if at all. The weak antagonistic activity of this variant might be due to its weaker affinity for the CNTFRα/gp130 complex, as compared with that of CNTF for the tripartite CNTFRα/gp130/LIFR receptor complex (35). A more potent inhibitor of CNTF action was generated by combining the antagonistic mutations with the S166D/Q167H (DH) substitutions, which confer a 40-fold increase in CNTFRα affinity (18). The resulting protein, AADH-CNTF, behaved as a potent competitive antagonist on both soluble and membrane-bound CNTF receptors. A similar potentiating effect on antagonistic activity has been previously reported for IL-6 variants with enhanced α-receptor binding (6, 36). Consistent with its specificity for CNTFRα, AADH-CNTF did not inhibit the effects of LIF or IL-6 in HepG2 cells. Because the antagonist does induce the formation of a complex containing CNTFRα and gp130, it might be able to inhibit the action of other gp130-utilizing cytokines in cells expressing CNTFRα and limiting amounts of the common signal transducer. Further experiments are needed to test this possibility.

The pleiotropic actions of CNTF and related cytokines may be due in part to the sharing and cross-activation of receptor subunits among the members of this cytokine family (2–4). For instance, CNTF can activate the LIF receptor (35), and an additional ligand(s) may signal through CNTFRα (37). The availability of CNTFRα-selective agonists and antagonists will be useful to dissect the role of this receptor subunit in the mechanism of action of CNTF and related cytokines.

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