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Agonistic and Antagonistic Variants of Ciliary Neurotrophic Factor (CNTF) Reveal Functional Differences between Membrane-bound and Soluble CNTF α-Receptor*

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Annalise Di Marco, Isabelle Gloaguen, Anna Demartis, Isabella Saggio‡, Rita Graziani, Giacomo Paonessa, and Ralph Laufer§

From the Istituto di Ricerche di Biologia Molecolare P. Angeletti (IRBM), Via Pontina km 30.600, 00040 Pomezia, Rome, Italy

Ciliary neurotrophic factor (CNTF) drives the sequential assembly of a receptor complex containing the ligand-specific α-receptor subunit (CNTFR) and the signal-transducing β-subunits gp130 and leukemia inhibitory factor receptor-β (LIFR). CNTFR can function in either membrane-bound or soluble forms. The membrane-bound form mediates the neuronal actions of CNTF, whereas the soluble form serves to confer cytokine responsiveness to non-neuronal cells expressing gp130 and LIFR. The objective of this work was to analyze whether the two receptor isoforms differ in their ability to interact functionally with CNTF and related proteins. Two new types of CNTF variants, characterized by weakened interactions with either CNTFR or both LIFR and gp130, were developed, and the biological activities of these and other mutants were determined in non-neuronal *versus* neuronal cells, as well as in non-neuronal cells transfected with an expression vector for CNTFR. Membrane anchoring of CNTFR was found to render the CNTF receptor complex relatively insensitive to changes in agonist affinity for either α- or β-receptor subunits and to promote a more efficient interaction with a gp130-depleting antagonistic variant of CNTF. As a result of this phenomenon, which can be rationalized in terms of the multivalent nature of CNTF receptor interaction, CNTF variants display striking changes in receptor selectivity.

Ciliary neurotrophic factor (CNTF)[1] is a protein expressed in glial cells, which stimulates gene expression and cell survival and differentiation in a variety of neuronal cell populations and has been proposed to act as a lesion factor involved in the prevention of nerve degeneration after injury (1, 2). In addition to its neuronal actions, CNTF can also elicit biological effects in non-neuronal cells, such as glia (3, 4), hepatocytes (5), skeletal muscle cells (6), embryonic stem cells (7), bone marrow stromal cells (8), and tumor plasma cells (9).

CNTF exerts its biological actions through the binding, sequential assembly, and activation of a multisubunit receptor complex composed of a ligand-specific α-receptor (CNTFR) and the signal-transducing β-subunits gp130 and leukemia inhibitory factor receptor-β (LIFR) (2, 10). Binding of CNTF to CNTFR triggers the subsequent association and heterodimerization of gp130 and LIFR, leading to the activation of a signaling cascade mediated by protein tyrosine kinases of the Janus kinase family and STAT transcription activators (2, 10). Similar to gp80, the α-receptor for IL-6, which mediates homodimerization of gp130 (10), CNTFR can function in either membrane-bound or soluble forms. The membrane-bound form of CNTFR (mb-CNTFR), which is anchored to the cell surface via a glycosylphosphatidylinositol linkage, is expressed in neuronal and skeletal muscle cells (11, 12). The soluble form of CNTFR (s-CNTFR), which can be produced by phospholipase C-mediated cleavage of mb-CNTFR, serves as a cofactor in potentiating CNTF actions on cells that express gp130 and LIFR (13). Soluble CNTFR has been detected in cerebrospinal fluid and serum (6, 13), suggesting that it may be involved in mediating some of the non-neuronal actions of CNTF such as hepatic acute phase response (14).

To modulate the receptor specificity of CNTF, we previously developed variants containing amino acid substitutions within specific sites of interaction with either CNTFR or LIFR-β. As expected, amino acid substitutions that increase the affinity for CNTFR ($CNTFR^+$) gave rise to CNTF variants with enhanced biological activity (15), whereas amino acid substitutions that impair the interaction with LIFR ($LIFR^-$) gave rise to variants with partial agonistic or antagonistic activities (16). An unexpected result was that a human neuroblastoma cell line expressing mb-CNTFR was much less sensitive to changes in CNTF affinity for CNTFR or LIFR than non-neuronal (hepatoma) cells responding to the combination of CNTF and s-CNTFR. To analyze this phenomenon further, we engineered two new types of CNTF variants, characterized by weakened interactions with either CNTFR ($CNTFR^-$) or both LIFR-β and gp130 ($LIFR^-$/$gp130^-$) and tested their biological activities in cells devoid of or expressing mb-CNTFR. The results of this study show that anchoring of CNTFR in the cell membrane renders the tripartite receptor complex relatively resistant to changes in ligand affinity which greatly affect bioactivity mediated through s-CNTFR. As a result of this phenomenon, CNTF variants with altered receptor interaction display striking changes in receptor selectivity.

EXPERIMENTAL PROCEDURES

CNTF Variants

D30Q/S166D/Q167H/Human CNTF (QDH-CNTF)—A point mutation giving rise to the D30Q substitution was introduced into the pRSET-DH-CNTF vector (15) by inverse polymerase chain reaction (17), yielding the bacterial expression vector pRSET-QDH-CNTF.

D30Q/F152A/S166D/Q167H/Human CNTF (QAKDH-CNTF)—

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‡ Present address: Dept. of Genetics and Molecular Biology, University of Rome La Sapienza, 00185 Rome, Italy.

§ To whom correspondence should be addressed. Tel.: 39-6-91093-440; Fax: 39-6-91093-225.

[1] The abbreviations used are: CNTF, ciliary neurotrophic factor; CNTFR, CNTF α-receptor; gp, glycoprotein; LIF, leukemia inhibitory factor; LIFR, LIF receptor-β; IL, interleukin; mb-, membrane-bound; s-, soluble; STAT, signal transducer and activator of transcription.

TABLE I
Human CNTF variants with altered receptor interaction

Mutation	Abbreviation	Site of mutation[a]	Receptor interaction[b]	Refs.
S166D/Q167H	DH-CNTF	1	$CNTFR^+$	15
T169I/H174A	IA-CNTF	1	$CNTFR^-$	This work
D3OQ		2	$gp130^-$	28
F152A	AK-CNTF	3	$LIFR^-$	16
D3OQ/S166D/Q167H	QDH-CNTF	1 + 2	$CNTFR^+/gp130^-$	This work
F152A/S166D/Q167H	AKDH-CNTF	1 + 3	$CNTFR^+/LIFR^-$	16
D3OQ/F152A/5166D/Q167H	QAKDH-CNTF	1 + 2 + 3	$CNTFR^+/LIFR^-/gp130^-$	This work

[a] Sites 1, 2, and 3 denote binding sites for CNTFR, gp130, and LIFR, respectively.
[b] The suffixes + and − denote increased and decreased receptor binding, respectively.

The pRSET-QDH-CNTF plasmid was digested with *Hin*dIII (which removes nucleotides 234–600 of the QDH-CNTF coding sequence) and ligated with the *Hin*dIII-*Hin*dIII fragment from pRSET-AKDH-CNTF (16), yielding pRSET-QAKDH-CNTF.

T169I/H174A/Human CNTF (IA-CNTF)—Mutations were generated by overlap extension polymerase chain reaction (18) using the pHenΔ-CNTF vector (19) as template. The resulting IA-CNTF coding sequence was subcloned into the pRSET-5d vector (20), using procedures described previously (15).

Recombinant proteins were produced in *Escherichia coli* and purified by reverse-phase HPLC according to procedures described previously (15, 16).

Generation of HepG2 Transfectants Stably Expressing mb-CNTFR

Human cDNA encoding the full-length human CNTFR (nucleotides 264–1382 coding for amino acids 1–372 (11)) was obtained by reverse transcription-polymerase chain reaction from SH-SY5Y cells (21) and cloned into the *Eco*RV site of the eukaryotic expression plasmid pcDNA3 (Invitrogen), which carries the neomycin resistance gene. DNA (20 μg) was transfected into HepG2 cells as a calcium phosphate precipitate (22), and cells were subjected to selection in complete culture medium (minimal essential medium containing penicillin, streptomycin, and 10% fetal calf serum) supplemented with 1 mg/ml G418. A subclone stably expressing CNTFR (HepG2/CNTFR) was identified on the basis of CNTF surface binding and CNTF-induced stimulation of haptoglobin production. HepG2/CNTFR cells were maintained in complete culture medium supplemented with 0.2 mg/ml G418.

CNTFR Binding Assay

The ability of CNTF variants to compete with biotinylated CNTF for binding to s-CNTFR was determined in a solid phase binding assay as described previously (15, 19).

Bioassays

Stimulation of haptoglobin production in HepG2 cells and of choline acetyltransferase activity in IMR-32 cells were determined as described previously (16).

Electromobility Shift Assay

HepG2 and HepG2/CNTFR cells were plated in 100-mm dishes and used 24 h later, when semiconfluent. Cells were deprived of serum for 4 h before a 15-min treatment with various effectors, as specified under "Results." The cells were then washed with ice-cold phosphate-buffered saline containing 50 mM NaF, collected by centrifugation, and frozen in liquid nitrogen. Total cell extracts were prepared as described previously (23). Binding of activated STAT factors to the high affinity SIE m67 oligonucleotide (24) was determined by electromobility shift assays according to Sadowski and Gilman (25), using 10 μg of cell extract. The oligonucleotide probe was labeled by filling in 5′-protruding ends with Klenow enzyme in the presence of [α-^{32}P]dATP and [α-^{32}P]dCTP (3,000 Ci/mmol). Complexes were resolved on 5% polyacrylamide, 2.5% glycerol, 0.5 × TBE (45 mM Tris borate, 0.5 mM EDTA, pH 7.8) gels, which were then dried and subjected to autoradiography.

RESULTS

Generation of $CNTFR^-$ and $LIFR^-/gp130^-$ Variants of CNTF—CNTF can trigger biological responses either through a low affinity interaction with the LIF receptor complex (LIFR + gp130) or through a high affinity interaction with the tripartite CNTF receptor complex (CNTFR + LIFR + gp130) (13, 26). The interactions of CNTF with its receptor subunits are thought to be mediated by distinct binding sites on the surface of the cytokine. Binding sites for CNTFR (site 1), gp130 (site 2), and LIFR (site 3) have been proposed to include residues in the D helix and A/B loop (15, 27), the A helix (28), and the D1 motif at the beginning of helix D, respectively (16, 29). Specific amino acid substitutions within these regions have been described which give rise to CNTF variants with increased affinity for CNTFR ($CNTFR^+$) and/or decreased affinity for gp130 ($gp130^-$) or LIFR ($LIFR^-$), as summarized in Table I.

In the present work, we introduced two new amino acid substitutions in helix D (30) of CNTF, which result in a $CNTFR^-$ variant, T169I/H174A/human CNTF (IA-CNTF) with ~15-fold reduced affinity for CNTFR (Fig. 1). To generate $CNTFR^+/gp130^-$ and $CNTFR^+/LIFR^-/gp130^-$ variants, we introduced the D30Q site 2 substitution, which was reported to decrease the interaction of CNTF with gp130 (28), into DH-CNTF (a $CNTFR^+$ variant) and AKDH-CNTF (a $CNTFR^+/LIFR^-$ variant), yielding D30Q/S166D/Q167H/human CNTF (QDH-CNTF), and D30Q/F152A/S166D/Q167H/human CNTF (QAKDH-CNTF), respectively (see Table I). The CNTFR binding affinity of QDH-CNTF and QAKDH-CNTF is slightly (~2-fold) lower than that of the parent DH-CNTF and ~10-fold higher than that of wild-type CNTF (Fig. 1). The expected reduced interaction of these proteins with gp130 should be reflected by a decreased ability to activate LIF receptors as well as CNTF receptors.

We first measured the ability of CNTF variants to stimulate haptoglobin secretion in the human hepatoma cell line HepG2, which expresses LIFR and gp130 but not CNTFR (31). CNTF activity in this system is thought to be mediated through stimulation of the LIFR complex (31). In this assay, wild-type CNTF is equipotent with the $CNTFR^+$ variant DH-CNTF (15) and also with the $CNTFR^-$ variant IA-CNTF (Fig. 2). QDH-CNTF was significantly less potent than the parent DH-CNTF, consistent with the detrimental effect of the D30Q substitution on gp130 binding (28). As reported previously (16), the $LIFR^-$ variant AKDH-CNTF displayed only marginal activity in this assay. Introduction of the D30Q substitution into this variant (QAKDH-CNTF) resulted in a complete loss of potency at concentrations up to 10 μg/ml (Fig. 2).

Biological Activity Mediated through s-CNTFR—Addition of s-CNTFR to HepG2 cells results in a dose-dependent reduction of the EC_{50} for CNTF or CNTF variants because of the formation of high affinity CNTF receptor complexes. A limiting EC_{50}, which does not depend on ligand affinity for CNTFR, is reached at high concentrations of s-CNTFR, reflecting cytokine saturation with the soluble receptor (16). Under these conditions, the EC_{50} depends solely on the affinity of the cytokine for LIFR and gp130. It follows that for CNTF variants with altered affinity for CNTFR, changes in biological activity will be apparent only at subsaturating concentrations of s-CNTFR (16). Conversely, the effects of mutations that affect the affinity of CNTF for LIFR or gp130 are best evaluated in the presence of saturating concentrations of s-CNTFR.

The biological activity of the $CNTFR^-$ variant IA-CNTF is

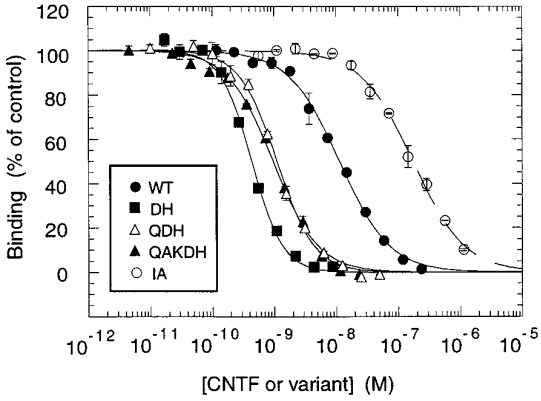

FIG. 1. **CNTFR binding of CNTF variants.** Binding of biotinylated human CNTF to immobilized CNTFR was determined in the absence (control) or presence of CNTF (●), IA-CNTF (○), DH-CNTF (■), QDH-CNTF (△), or QAKDH-CNTF (▲). Results are expressed as percent of control binding and represent the mean ± S.D. from duplicate determinations. Data are from a representative experiment that was repeated three times with similar results.

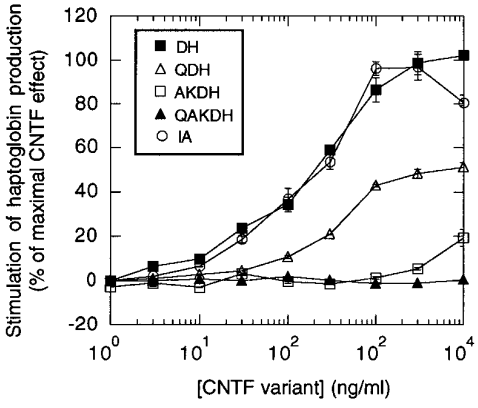

FIG. 2. **LIFR-mediated biological activity of CNTF variants in HepG2 cells.** The following proteins were tested for their ability to stimulate haptoglobin production in HepG2 cells: IA-CNTF (○), DH-CNTF (■), QDH-CNTF (△), AKDH-CNTF (□), and QAKDH-CNTF (▲). For clarity, the effect of CNTF, which is equipotent with DH-CNTF in this assay (15), is not shown. Results are expressed as a percentage of the maximal CNTF-induced response. Each point is the mean ± S.E. from at least two separate experiments.

depicted in Fig. 3*A*. At a subsaturating concentration of s-CNTFR, this variant behaved as a full agonist in the HepG2 assay, with an EC_{50} value five times higher than that of CNTF, in agreement with its reduced affinity for CNTFR. The biological activity of the $CNTFR^+/LIFR^-/gp130^-$ variant QAKDH-CNTF was determined in the presence of a saturating concentration of s-CNTFR (*i.e.* under conditions of maximal potency). The previously reported (15, 16) activities of the $CNTFR^+$ and $CNTFR^+/LIFR^-$ variants DH-CNTF and AKDH-CNTF are shown for comparison (Fig. 3*B*). As reported (16), the impaired LIFR interaction of AKDH-CNTF is reflected by a 40-fold higher EC_{50} and 1.2-fold lower efficacy relative to DH-CNTF. QAKDH-CNTF displayed even weaker activity, with a 300-fold increased EC_{50} and 1.8-fold decreased efficacy with respect to DH-CNTF (Fig. 3*B*).

Biological Activity Mediated through mb-CNTFR—We next assessed the ability of CNTF variants to induce choline acetyltransferase in the human neuroblastoma cell line IMR-32, which expresses mb-CNTFR (16, 32). The previously reported (16) activities of DH-CNTF (which displays the same activity as CNTF in this system) and AKDH-CNTF are shown for comparison (Fig. 4*A*). IA-CNTF was equipotent with DH-CNTF, whereas QAKDH-CNTF behaved as a partial but potent agonist in IMR-32 cells, with an unchanged EC_{50} and 1.5-fold reduced efficacy relative to DH-CNTF. These results extend our previous observation that the mb-CNTFR of IMR-32 cells is not very sensitive to changes in CNTF affinity for either α-receptor or β-receptor subunits (16).

A possible explanation for this phenomenon is that high local concentrations of mb-CNTFR can partially counteract changes in ligand-receptor interaction. If so, a decrease in the concentration of mb-CNTFR should reveal potency differences among CNTF variants with differing receptor affinities. The CNTF receptor antagonist AADH-CNTF, which binds potently to CNTFR but not to LIFR (16), was used to reduce the free concentration of mb-CNTFR on the surface of IMR-32 cells. As depicted in Fig. 4*B*, this treatment led to a 20-fold increase in the relative potency (*versus* CNTF) of the $CNTFR^+$ variant DH-CNTF and a 5-fold decrease in the relative potency of the $CNTFR^-$ variant IA-CNTF. Furthermore, in the presence of AADH-CNTF, the relative potency (*versus* DH-CNTF) of AKDH-CNTF was decreased 40-fold, whereas QAKDH-CNTF was inactive up to a concentration of 1 μg/ml (Fig. 4*C*). Thus, after a pharmacological treatment that reduces the concentration of free mb-CNTFR in IMR-32 cells, the order of potencies of CNTF variants resembled that observed in non-neuronal cells in the presence of exogenous s-CNTFR (see Fig. 3).

Membrane-bound CNTFR Counteracts Changes in CNTF Receptor Affinity—The foregoing results support the notion that high local concentrations of mb-CNTFR can normalize the biological activities of CNTF variants with greatly differing affinities for CNTF receptor subunits. To ensure that this is not a peculiar feature of IMR-32 cells, we asked whether a similar effect would be obtained in HepG2 cells stably transfected with an expression vector encoding full-length CNTFR (HepG2/CNTFR). The presence of functional mb-CNTFR in HepG2/CNTFR cells was confirmed by the ability of CNTF to rapidly induce the activation of STAT transcription factors in the absence of s-CNTFR (Fig. 5). In contrast, STAT activation by CNTF in HepG2 cells required the presence of s-CNTFR. CNTF induced a similar pattern of DNA binding-competent STAT factors in the two cell lines, which was indistinguishable from that of LIF, namely a majority of DNA-binding STAT3 homodimers and a smaller amount of STAT1 homodimers and STAT1·STAT3 heterodimers (Fig. 5).

Fig. 6 depicts the dose-response relationships for the stimulation of haptoglobin production by CNTF variants in HepG2/CNTFR cells. The dose-response curves in this system were bell-shaped, as also observed in experiments with some batches of IMR-32 cells (data not shown). The reason for this phenomenon is not known, but it could be related to the ability of CNTF to form multimeric nonsignaling subcomplexes with CNTFR and either LIFR or gp130 (33). The order of agonist potencies was similar to that obtained in IMR-32 cells. Thus, CNTF (not shown), DH-CNTF, and IA-CNTF displayed comparable biological activities. Both AKDH-CNTF and QAKDH-CNTF elicited lower maximal effects (1.2- and 1.8-fold, respectively), and QAKDH-CNTF also displayed a higher EC_{50} value (5-fold) relative to DH-CNTF (Fig. 6). Yet, the activities of both partial agonists were significantly higher in HepG2/CNTFR cells than in the parent HepG2 cells assayed in the presence of saturating concentrations of s-CNTFR. For comparison, the relative potencies of AKDH-CNTF and QAKDH-CNTF *versus* DH-CNTF (EC_{50} ratios) were 35-fold and 60-fold higher, respectively, in HepG2/CNTFR cells than in HepG2 cells with saturating s-CNTFR (see Fig. 3*B*). These results demonstrate that the different pharmacological profiles of CNTF variants in non-neuronal *versus* neuronal cells are caused by a functional difference between soluble *versus* membrane-bound CNTFR.

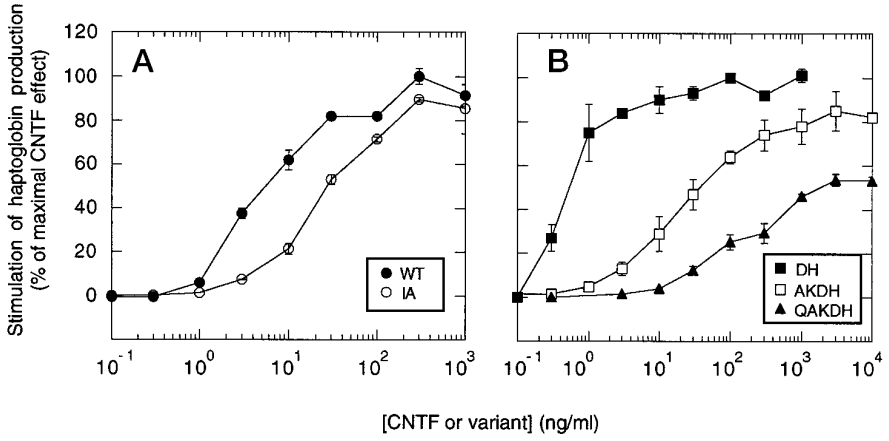

FIG. 3. **s-CNTFR-mediated biological activity of CNTF variants in HepG2 cells.** Stimulation of haptoglobin production in HepG2 cells was determined in the presence of 80 ng/ml (*panel A*) or 800 ng/ml (*panel B*) s-CNTFR. The proteins tested were: CNTF (●), IA-CNTF (○), DH-CNTF (■), AKDH-CNTF (□), and QAKDH-CNTF (▲). In *panel B*, the effect of CNTF, which is equipotent with DH-CNTF under these conditions (16), is not shown for clarity. Treatment of results was as described in the Fig. 2 legend.

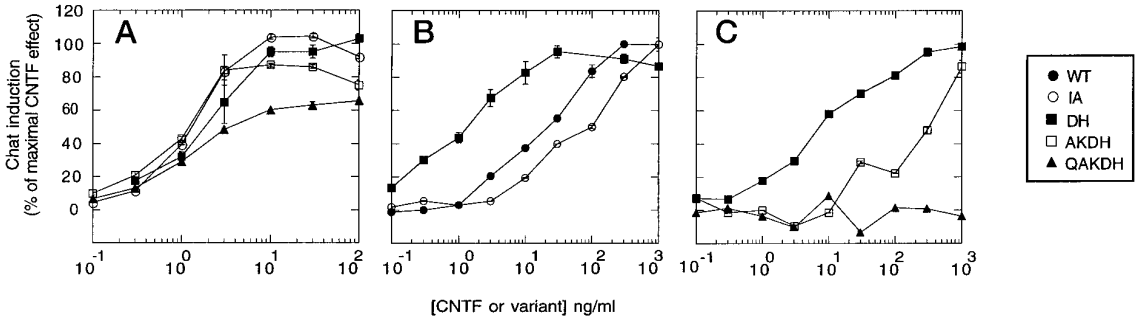

FIG. 4. **mb-CNTFR-mediated biological activity of CNTF variants in IMR-32 cells.** Induction of choline acetyltransferase (*Chat*) activity in IMR-32 cells was determined either in the absence (*panel A*) or presence of 1 μg/ml (*panel B*) or 10 μg/ml (*panel C*) of the CNTF receptor antagonist AADH-CNTF. The proteins tested were CNTF (●), IA-CNTF (○), DH-CNTF (■), AKDH-CNTF (□), and QAKDH-CNTF (▲). In *panel A*, the effect of CNTF, which is equipotent with DH-CNTF in this assay (16), is not shown for clarity. Results are expressed as a percentage of the maximal CNTF-induced response. Each point is the mean ± S.E. from at least two separate experiments (*panel A*) or from single representative experiments performed in duplicate culture dishes (*panels B* and *C*).

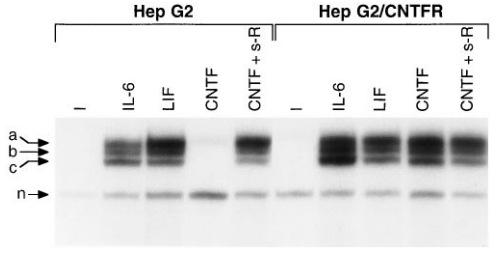

FIG. 5. **Early signaling responses mediated by the combination of CNTF + s-CNTFR in HepG2 cells and CNTF in HepG2/CNTFR cells.** Cells were either not treated (−) or treated for 15 min with 100 ng/ml IL-6, LIF, CNTF, or 100 ng/ml s-CNTFR plus 100 ng/ml CNTF (*CNTF + s-R*). Activation of cellular STAT factors was determined by electromobility shift assay. *Arrows* denote the positions of migration of bound STAT3 homodimers (*a*), STAT1·STAT3 heterodimers (*b*), and STAT1 homodimers (*c*) (46). *n*, nonspecific binding.

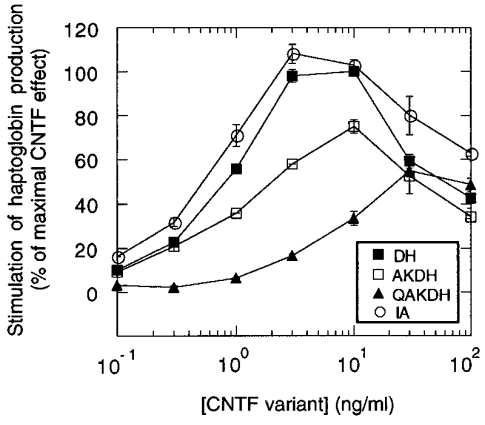

FIG. 6. **mb-CNTFR-mediated biological activity of CNTF variants in HepG2/CNTFR cells.** Experimental details and treatment of results were as described in the Fig. 2 legend. The proteins tested were IA-CNTF (○), DH-CNTF (■), AKDH-CNTF (□), and QAKDH-CNTF (▲). CNTF (not shown) is equipotent with DH-CNTF in this assay.

The presence of mb-CNTFR Alters the Pharmacological Specificity of a CNTF Receptor Antagonist—The different abilities of soluble *versus* membrane-bound CNTFR to mediate the activities of CNTF receptor agonists raised the question of whether these receptors might also differ in their interaction with a CNTF receptor antagonist. AADH-CNTF is unable to bind LIFR, but it can form a stable complex with CNTFR and gp130. As reported previously (16), AADH-CNTF, in the presence of s-CNTFR, specifically inhibited the action of CNTF in HepG2 cells without affecting that of other gp130-utilizing cytokines, such as LIF or IL-6 (Fig. 7*A*). In contrast, the antagonist was able to inhibit the responses to all three cytokines in cells expressing mb-CNTFR, such as HepG2/CNTFR (Fig. 7*B*) and IMR-32 (Fig. 7*C*). These results suggest that mb-CNTFR, but not s-CNTFR, can efficiently promote the formation of a complex composed of AADH-CNTF, mb-CNTFR, and gp130, leading to depletion of the signal transducer.

DISCUSSION

The results of the present study demonstrate that membrane-bound and soluble CNTFR differ functionally in their ability to mediate the agonistic or antagonistic actions of CNTF variants. Thus, membrane anchoring of CNTFR renders the CNTF receptor complex relatively insensitive to changes in agonist affinity for either α- or β-receptor subunits and promotes a more efficient interaction with a gp130-depleting antagonistic variant. That these effects are caused by mb-CNTFR

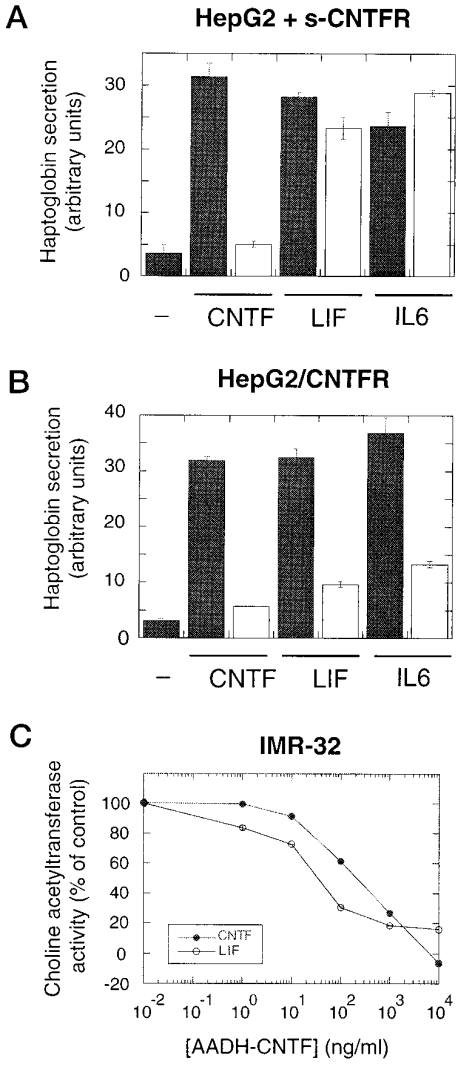

FIG. 7. **Inhibition of gp130-mediated cytokine responses by AADH-CNTF in cells expressing mb-CNTFR.** Cytokine-induced responses were determined in HepG2 cells in the presence of 800 ng/ml s-CNTFR (*panel A*), HepG2/CNTFR cells (*panel B*), or IMR-32 cells (*panel C*). HepG2 cells were either not treated with any cytokine (−) or treated with 1 ng/ml CNTF, LIF, or IL-6 in the absence (*filled bars*) or presence (*empty bars*) of 10 μg/ml AADH-CNTF. Choline acetyltransferase induction by 3 ng/ml CNTF or LIF in IMR-32 cells was determined in the absence (control) or presence of increasing concentrations of AADH-CNTF.

rather than being an intrinsic feature of the cells expressing it is borne out by the following line of evidence.

CNTF variants with greatly differing affinities for CNTFR, LIFR, or gp130 displayed corresponding changes in s-CNTFR-mediated biological activity in the non-neuronal HepG2 cell line but were almost equipotent in stimulating mb-CNTFR-mediated responses in the neuronal IMR-32 cell line. Treatment of IMR-32 cells with the competitive antagonist AADH-CNTF (16), which reduces the concentration of mb-CNTFR and gp130 available for agonist binding, uncovered potency differences among CNTF variants which paralleled their altered interactions with CNTF receptor subunits (see Fig. 4, *B* and *C*). Inasmuch as AADH-CNTF does not interact with LIFR, this finding rules out the possibility (16) that the particular profile of agonist potencies in IMR-32 cells is the result of high concentrations of LIFR. The effect of the antagonist could be caused in part by its ability to deplete gp130 (see below). In particular, this would explain the strong antagonist-mediated inhibition of biological activity observed in the case of the gp130$^-$ variant QAKDH-CNTF (see Fig. 4*C*). Yet, gp130 is unlikely to influence the relative biological activities of CNTF variants such as IA-CNTF, DH-CNTF, and AKDH-CNTF, which differ from the wild-type protein only in their affinities for CNTFR or LIFR. Thus, the antagonist-mediated shift in the relative biological activities of these ligands is most likely caused by a reduction in the concentration of free mb-CNTFR, suggesting that high amounts of the latter are responsible for the equipotency of CNTF variants in IMR-32 cells. Strong support for this notion comes from the finding that expression of full-length mb-CNTFR was sufficient to confer to HepG2 cells the characteristic profile of agonist and antagonist potencies observed in neuronal cells expressing mb-CNTFR.

What could be the mechanism underlying the functional differences between mb-CNTFR and s-CNTFR? Inasmuch as mb-CNTFR has no cytoplasmic domain (11) and is not known to interact directly with intracellular mediators, it is unlikely that the two receptor isoforms differ intrinsically in their signal transduction capabilities. Indeed, the immediate-early signaling events induced by CNTF in cells bearing mb-CNTFR are indistinguishable from those induced by the combination of CNTF and s-CNTFR (13) (see also Fig. 5). One possibility considered previously (16) is that the two receptor isoforms differ in their ability to mediate ligand-induced receptor internalization (34), which could result in different kinetics of agonist action (35). Such a mechanism would not, however, explain the different interactions of mb-CNTFR and s-CNTFR with an antagonistic CNTF variant observed in the present work.

The ligand retention hypothesis (36, 37) provides a plausible explanation for the pharmacological behavior of cytokine variants with membrane-bound and soluble receptor isoforms. As pointed out by Baumann and co-workers (36), high receptor concentrations at the cell surface (see below) can lead to near unidirectional ligand capture. This would explain why cytokine variants with altered receptor binding affinity can display unchanged agonistic potencies, as reported for growth hormone (38), IL-6 (39), and CNTF (Ref. 16 and present work). The equipotency of CNTFR$^-$ and CNTFR$^+$ variants in IMR-32 cells would thus be the result of quasi-irreversible ligand capture by mb-CNTFR, analogous to the situation in HepG2 cells in the presence of saturating concentrations of s-CNTFR (16).

The finding that mb-CNTFR is able to equalize the biological activities of agonists with greatly differing α- or β-receptor interaction can be understood considering the multivalent nature of CNTF binding to the different subunits of its tripartite receptor. Receptor binding kinetics of multivalent ligands, such as antibodies (40, 41), epidermal growth factor (42), and growth hormone (36), have been analyzed previously in great detail. For the purpose of the present discussion, we will consider a simple cross-linking model, in which one molecule of CNTF (L) sequentially binds to CNTFR (R_1), gp130 (R_2), and LIFR (R_3). Formation of the tripartite receptor complex ($LR_1R_2R_3$) can be described by the following linked equilibria

$$L + R \Leftrightarrow LR_1 \quad K_1 = [LR_1]/[L][R] \qquad \text{(Eq. 1)}$$

$$LR_1 + R_2 \Leftrightarrow LR_1R_2 \quad K_2 = [LR_1R_2]/[LR_1][R_2] \qquad \text{(Eq. 2)}$$

$$LR_1R_2 + R_3 \Leftrightarrow LR_1R_2R_3 \quad K_3 = [LR_1R_2R_3]/[LR_1R_2][R_3] \qquad \text{(Eq. 3)}$$

where K_1, K_2, and K_3 are the affinity constants for each step. The concentration of the functional CNTF-bound tripartite receptor complex, which determines biological activity, is given by Equation 4.

$$[LR_1R_2R_3] = K_1K_2K_3[R_1][R_2][R_3][L] \qquad \text{(Eq. 4)}$$

By analogy to the model of multivalent antibody binding developed by Dower *et al.* (41), at the limit of low receptor occupancy

the affinity of ligand for R_3, once it is already bound to R_1 and R_2, is given by $K_1K_2K_3[R_1]_0[R_2]_0$, with $[R_n]_0$ denoting total receptor concentration in the membrane. Human neuroblastoma cells express 500–1000 high affinity sites and 10,000–40,000 low affinity sites for CNTF (26, 43). Assuming equal amounts of LIFR and gp130 (corresponding to the number of high affinity sites) and an effective volume for receptor interaction corresponding to a 100 Å shell about spherical cells of radius 10 μm (36, 41), the concentrations of R_1, R_2, and R_3 on the surface of human neuroblastoma cells can be estimated to be $\approx$ 1–5 $\times$ 10^{-6} M, 0.5–1 $\times$ 10^{-7} M, and 0.5–1 $\times$ 10^{-7} M, respectively. The binding constant for trimerically bound CNTF in human neuroblastoma cells is in the order of 10^{10} M^{-1} (26, 43). Thus, the local concentration of CNTF receptor β-subunits is $\approx$ 1,000-fold higher than the equilibrium dissociation constant for CNTF. Under these conditions, ligand will be effectively trapped into the tripartite complex (36). For the $CNTFR^+$ variants used in the present work, K_1 is $\approx$ 20-fold higher than for wild-type, which will give rise to a greater than 10,000-fold ratio between β-receptor concentration and ligand equilibrium dissociation constant. It can therefore be expected that in this system, only variants of DH-CNTF whose affinity for LIFR and/or gp130 is reduced by more than 4 orders of magnitude will display decreased tripartite receptor binding with respect to wild-type. The present results indicate that this is not the case for AKDH-CNTF and QAKDH-CNTF, even though the affinity of these variants for the LIFR is too low to be detectable in direct binding experiments using soluble receptor subunits (16).

In contrast to mb-CNTFR, increasing concentrations of s-CNTFR in HepG2 cells can potentiate the biological activities of partial agonists only up to a maximal limit, which is still much lower than that of the parent analogs with wild-type β-receptor affinity (see Fig. 3*B*). According to the model discussed above, this would be simply because the effective affinity constant is proportional to $[R_1]_0$, which is $\approx$ 1,000-fold higher for m-CNTFR (10^{-6} M) than for a concentration of s-CNTFR near to saturation of DH-CNTF binding (10^{-9} M). Further increasing the amount of s-CNTFR will lead to ligand depletion ([L] $\rightarrow$ 0) without significantly increasing the overall affinity. Thus, the effective affinity constants for formation of the complexes between CNTF or DH-CNTF with the tripartite receptor will be $\approx$ 1,000-fold lower than for m-CNTFR, *i.e.* in the order of 10^7 M^{-1} and 10^8 M^{-1}, respectively. HepG2 cells express $\approx$ 200 (LIFR + gp130) binding sites (44). Assuming that this is equal to the amount of LIFR, the membrane concentration of the latter is $\approx$ 10^{-8} M. Under these conditions, irreversible ligand trapping into the tripartite receptor complex will not occur, and changes in K_2 or K_3 will be reflected by a decrease in ligand binding. It should be noted that all of the above calculations are based on rough estimates of binding constants and receptor concentrations and merely serve to illustrate that a simple model of multivalent receptor binding can account for the results of the present work.

The ability of mb-CNTFR but not s-CNTFR to mediate inhibition of IL-6 and LIF signaling by the CNTF receptor antagonist AADH-CNTF can also be rationalized in terms of a local concentration effect. Since AADH-CNTF is mutated only in the site of interaction with LIFR (site 3) but not with gp130 (site 2) we hypothesized previously that it might be able to deplete gp130 in cells expressing mb-CNTFR and limiting amounts of the signal transducer (16). A similar mechanism is likely to be responsible for the inhibition of IL-11 activity by site 2 IL-6 antagonists that can bind only one out of the two gp130 molecules required for the formation of a functional receptor complex (45). The present finding that mb-CNTFR is necessary and sufficient to confer gp130-antagonistic properties to AADH-CNTF supports the notion that membrane capture of ligand-CNTFR complexes is required to stabilize their interaction with β receptor subunits.

On the basis of the present results it can be anticipated that AADH-CNTF will behave as a specific antagonist of other CNTF responses mediated through s-CNTFR while serving as a "general" gp130 antagonist in cells expressing high membrane concentrations of CNTFR. Theoretically, it should be possible to improve the CNTF receptor selectivity of the antagonist by abolishing its ability to interact with gp130. This approach was applied successfully to the generation of selective IL-6 receptor antagonists with mutations in both sites 2 and 3 (45). Preliminary results[2] indicate that introduction of the D30Q site 2 mutation into AADH-CNTF is not sufficient to abolish its gp130-inhibiting activity. This is probably because of residual gp130 binding, consistent with the result that agonistic variants bearing the D30Q mutations retain significant biological activity in cells expressing mb-CNTFR. Further structure-function studies will be necessary to identify amino acid residues in site 2 whose substitution will completely abolish the interaction of CNTF with gp130.

In contrast to wild-type CNTF, which does not discriminate between membrane-bound and soluble CNTFR, CNTF agonists with impaired β-receptor interaction display receptor selectivity. For instance, QAKDH-CNTF is more than 30-fold more potent in IMR-32 or HepG2/CNTFR cells than in HepG2 cells with added s-CNTFR. Since CNTFR is expressed predominantly in neurons (12), enhanced selectivity for mb-CNTFR would be expected to translate into more neuron-specific pharmacological actions *in vivo*. Experiments are in progress to test this hypothesis.

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