The Amino Acid Exchange R28E in Ciliary Neurotrophic Factor (CNTF) Abrogates Interleukin-6 Receptor-dependent but Retains CNTF Receptor-dependent Signaling via Glycoprotein 130 (gp130)/Leukemia Inhibitory Factor Receptor (LIFR)

Background: CNTF signaling is mediated by CNTFR or IL-6R in complex with gp130 and LIFR. Results: The CNTFR variant CV-1 is CNTFR-selective. Conclusion: The single amino acid exchange R28E within CNTF abrogated IL-6R binding. Significance: CV-1 allows discrimination between CNTFR- and IL-6R-mediated effects in vivo.

Ciliary neurotrophic factor (CNTF) is a neurotrophic factor with therapeutic potential for neurodegenerative diseases. Moreover, therapeutic application of CNTF reduced body weight in mice and humans. CNTF binds to high or low affinity receptor complexes consisting of CNTFR/gp130-LIFR or IL-6R/gp130-LIFR, respectively. Clinical studies of the CNTF derivative Axokine revealed intolerance at higher concentrations, which may rely on the low-affinity binding of CNTF to the IL-6R. Here, we aimed to generate a CNTFR-selective CNTF variant (CV). CV-1 contained the single amino acid exchange R28E. Arg28 is in close proximity to the CNTFR binding site. Using molecular modeling, we hypothesized that Arg28 might contribute to IL-6R/CNTFR plasticity of CNTF. CV-2 to CV-5 were generated by transferring parts of the CNTF-binding site from cardiotrophin-like cytokine to CNTF. Cardiotrophin-like cytokine selectively signals via the CNTFR/gp130-LIFR complex, albeit with a much lower affinity compared with CNTF. As shown by immunoprecipitation, all CNTF variants retained the ability to bind to CNTFR. CV-1, CV-2, and CV-5, however, lost the ability to bind to IL-6R. Although all variants induced cytokine-dependent cellular proliferation and STAT3 phosphorylation via CNTFR-gp130-LIFR, only CV-3 induced STAT3 phosphorylation via IL-6R-gp130-LIFR. Quantification of CNTF-dependent proliferation of CNTFR-gp130-LIFR expressing cells indicated that only CV-1 was as biologically active as CNTF. Thus, the CNTFR-selective CV-1 will allow discriminating between CNTFR- and IL-6R-mediated effects in vivo.

The interleukin (IL)-6 type cytokine family consists of cardiotrophin-1, cardiotrophin-like cytokine (CLC), CNTF, IL-6, IL-11, IL-27, IL-30, IL-31, leukemia inhibitory factor (LIF), and oncostatin M (OSM) (1). Except for IL-31, all IL-6 type cytokines bind to the β-receptor gp130, demonstrating a high degree of binding site plasticity within gp130 toward the respective cytokines. Whereas IL-6 and IL-11 bind to homodimeric gp130 receptor complexes, all other cytokines of this family bind to heterodimeric signal-transducing β-receptor complexes, e.g. CNTF and CLC to gp130-LIFR (1). IL-6 type cytokine signaling activates the intracellular signaling cascades JAK/STAT, MAPK, and PI3K. For some IL-6 type cytokines, binding to their β-receptors is dependent on the initial complex formation with an α-receptor. The following high-affinity cytokine/α-receptor pairs exist: CLC/CNTFR, CNTF/CNTFR, IL-6/IL-6R, IL-11/IL-11R, and p28/Epselin-Barr virus-induced gene 3 (1). Moreover, the IL-6R was described as alternative low affinity α-receptor for CNTF and p28 (2,3), highlighting receptor plasticity also at the α-receptor/cytokine binding interface. IL-6 type cytokines have a typical four-helix bundle fold that links the four α-helices (A-D) by two long loops (A-B and C-D) and one short loop (B-C) placing the four helices in an up-up-down-down orientation. Here, site I of the cytokine binds to the α-receptor, whereas site II and site III are needed for the interaction with the β-receptors. Site I is constituted by the C-terminal A-B loop (site Ia) and the C-terminal D helix (site Ib), site II by parts of the A and C helix and site III by the C-terminal A helix and the N-terminal A-B loop (site IIIa), the B-C loop with adjacent parts of the B and C helix (site IIIb), and the C-terminal C-D loop with the adjoining NH2-terminal D helix (site IIIc).

Binding of IL-6 type cytokines to their receptors is mainly mediated by ionic and hydrophobic interactions (4). Close

**References**

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This article contains supplemental Fig. 1.

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Generation of a CNTFR-selective CNTF

A CNTFR-selective CNTF might be achieved by transfer of site I from the CNTFR-selective cytokine CLC to CNTF or by introduction of specific amino acid exchanges in CNTF. Importantly, because even though the binding to the low affinity site I from the CNTFR-selective cytokine CLC to CNTF or by viral IL-6, in which an extended site III of IL-6 is exchanged without the need of the IL-6Rα (10). IV-9 is a chimera of IL-6 and viral IL-6, in which an extended site III of IL-6 is exchanged by site III of viral IL-6. Consequently, IV-9 signaling via a gp130 homodimer was independent of the IL-6R (11).

The transfer of site I or II has, however, not been described as of yet. Moreover, abrogation of promiscuous cytokine/receptor usage by introduction of single point mutations within the cytokine or cytokine receptor has also not been achieved. Generation of CNTF or p28 variants specific for a single receptor would pave the way to analyze the function of CNTF/CNTFR versus CNTF/IL-6R-induced or p28/EBI3 versus p28/IL-6R-induced signal transduction in vivo.

A CNTFR-selective CNTF might be achieved by transfer of site I from the CNTFR-selective cytokine CLC to CNTF or by introduction of specific amino acid exchanges in CNTF. Importantly, because even though the binding to the low affinity α-receptor will be abrogated, this must not result in a major reduction of binding affinity of CNTF to the high-affinity CNTFR.

Here, we describe the development the human CNTF variant (CV)-1, which binds with high affinity to the CNTFR and induces signal transduction via LIFR and gp130. Due to the single point mutation R28E, interaction of CV-1 with the IL-6R was abrogated. CV-1 might be used to discriminate between IL-6R and CNTF-mediated effects of CNTF in vivo.

**EXPERIMENTAL PROCEDURES**

**Cells and Reagents**—Ba/F3-gp130 cells transduced with human gp130 were obtained from Immunix (Seattle, WA) (12). Ba/F3-gp130-hIL-6R cells (13), BaF/3-gp130-LIFR cells, BaF/3-gp130-LIFR-IL-6R, and BaF/3-gp130-LIFR-CNTFR cells were generated as described previously (9). HepG2 and COS-7 cells were from ATCC (Manassas, VA). All cells were grown in DMEM high-glucose culture medium (PAA Laboratories, Colbe, Germany) supplemented with 10% fetal bovine serum, penicillin (60 μg/ml), and streptomycin (100 μg/ml) at 37 °C with 5% CO₂ in a water-saturated atmosphere. All Ba/F3 cells were cultured using 10 ng/ml recombinant Hyper IL-6, which is a fusion protein of IL-6 and soluble IL-6R (sIL-6R) acts as a growth factor for Ba/F3-gp130 cells (14, 15). Hyper IL-6 was expressed and purified as described previously (14). Human LIF was purchased from R&D Systems (Minneapolis, MN). Phospho-STAT3 mAb (Tyr-705) and STAT3 mAb (124H6) were purchased from Cell Signaling Technology (Frankfurt, Germany). The peroxidase conjugated secondary antibodies were purchased from Pierce (Thermo Scientific). All restriction enzymes were obtained from Fermentas (Thermo Scientific).

**Cloning of CNTF, CV-1 to CV-5 and sCNTFR-Fc**—The bacterial expression plasmid pet23a (+)-CNTF (d14d15-C17A-Q63R-CNTF) coding for a N- and C-terminal shortened version of CNTF, including the amino acid exchanges C17A and Q63R was generated using standard PCR and mutagenesis procedures. pet23a (+)-CNTF and pet23a (+)-CLC were used to generate the expression plasmids pet23a (+)-CV-1 to pet23a (+)-CV-5 using site-directed mutagenesis (pet23a (+)-CV-1) and splicing by overlapping extension PCR (pet23a (+)-CV-2 to pet23a (+)-CV-5). Initially, PCR products were cloned into pPCR-Script Amp SK (+) and subcloned into pet23a (+) via NdeI and NotI restriction sites. The sequences of all primers used are available on request.

To generate a fusion protein of human soluble CNTFR (sCNTFR) with an Fc portion, the coding sequence of CNTFR (corresponding to amino acids 1–346) was amplified by PCR. In 5’ and 3’ of sCNTFR Sall BglII restriction sites were inserted (5’ primer: AAAGCGTCACGGCCTGTTGTTGTTGCTGCC and 3’ primer AAAGTACAAAGATCTGGGCTCTACGGG).

**Expression, Purification, and Renaturation of CV-1 to CV-5, CLC, IC-7, IL-6, and CNTF**—Expression of CV-1 to CV-5, CLC, IC-7, IL-6, and CNTF was performed in Escherichia coli BL21(DE3) (Merck KGaA). In detail, protein expression was induced at an A₆₀₀ of 0.8 with 1 mM isopropyl 1-thio-β-d-galactopyranoside. Cells were harvested after 4 h at 37 °C. The pelleted bacterial cells were lysed by sonication (Sonopuls HD2200, Bandelin, Berlin, Germany). CV-1 to CV-5, CLC, IC-7, and CNTF inclusion bodies were purified by repeated washing and sonication steps (three times) in 50 mM Tris-HCl, 0.2% Tween 20, pH 8.0, followed by washing with (three times) 50 mM Tris-HCl, pH 8.0. Purified inclusion bodies were dissolved in 6 M guanidine hydrochloride, 50 mM Tris-HCl, pH 8.0. Denatured CV-1 to CV-5, IC-7, IL-6, and CNTF were refolded at a protein concentration of 0.1 mg/ml by two-step 24-h dialysis against 50 mM Tris-HCl, pH 8.0, 1 M GuHCl, 3 M oxidized glutathione, 0.6 M reduced glutathione and against 50 mM Tris-HCl, pH 8.0, 3 mM reduced glutathione, and 0.9 mM oxidized glutathione. CNTF was refolded by dialysis against 50 mM CAPS, pH 11. Dialyzed CV-1 to CV-5, CLC, IC-7, IL-6, and CNTF were centrifuged for 30 min at 40,000 × g at 4 °C. CV-1 to CV-5, CLC, IC-7, IL-6, and CNTF were sterile filtered and concentrated with an Amicon stirred ultrafiltration cell.
equipped with an YM10 filter membrane, nominal molecular weight limit of 10,000 (Merck Millipore, Merck KGaA, Germany). CV-1 to CV-5, CLC, IC-7, IL-6, and CNTF were purified by size exclusion chromatography with an ÄKTAexplorer chromatography system and a HiLoad 16/60 Superdex 200 prep grade column (both from GE Healthcare). The fractions containing monomeric CV-1 to CV-5, CLC, IC-7, IL-6, and CNTF were concentrated by a Vivaspin 20 column, molecular weight cut-off of 10,000 (GE Healthcare). Pure CV-1 to CV-5, CLC, IC-7, IL-6, and CNTF proteins were analyzed by Coomasie Blue staining and Western blotting using a standard SDS-PAGE under reduced conditions using biotinylated mouse c-Myc (9E10) antibody (Santa Cruz Biotechnology). Folding of pure monomeric CV-1 to CV-5, CLC, IC-7, IL-6, and CNTF was verified by circular dichroism in a quartz cuvette (Helma, Mühlheim, Germany) with a Jasco J-720 CD-spectropolarimeter (Jasco Corp., Tokyo, Japan). The instrument was calibrated with an aqueous solution of 10-camphorsulfonic acid at 25 °C. The spectral bandwidth was 1.5 nm.

**Proliferation Assays**—The different transduced Ba/F3 cell lines were washed three times with sterile PBS and suspended in DMEM containing 10% FBS at a final concentration of 5 × 10^5 cells per well in a 96-well microtiter plate. Cells were incubated for 72 h as indicated with the cytokines and cytokine receptors in a final volume of 100 µl. After 72 h, cell growth was measured using the CellTiter Blue cell viability assay reagent (Promega, Karlsruhe, Germany) following the manufacturer’s instructions. The extinction was measured using a Lambda Fluoro 320 microplate fluorescence reader (excitation, filter 530/25; emission, filter 590/35; Software KC4, Bio-Tek Instruments). Normalization of relative light units was achieved by subtraction of negative control values. One representative example of two to three experiments was chosen for the figures. For clarity, no standard deviations were included in the diagrams; the S.D. were, as common for Ba/F3 cells, only minimally and usually below 5%. The different transduced Ba/F3 cell lines were washed three times with sterile PBS and suspended in DMEM containing 10% FBS at a final concentration of 5 × 10^5 cells per well in a 96-well microtiter plate. Cells were incubated for 72 h as indicated with the cytokines and cytokine receptors in a final volume of 100 µl. After 72 h, cell growth was measured using the CellTiter Blue cell viability assay reagent (Promega, Karlsruhe, Germany) following the manufacturer’s instructions. The extinction was measured using a Lambda Fluoro 320 microplate fluorescence reader (excitation, filter 530/25; emission, filter 590/35; Software KC4, Bio-Tek Instruments). Normalization of relative light units was achieved by subtraction of negative control values. One representative example of two to three experiments was chosen for the figures. For clarity, no standard deviations were included in the diagrams; the S.D. were, as common for Ba/F3 cells, only minimally and usually below 5%.

**Immunoprecipitation with sCNTFR-Fc and sIL-6R-Fc**—For co-precipitation, supernatants of p409DC-IL-6R-Fc and pDC-CNTFR-Fc transiently transfected COS-7 were collected. For co-precipitation supernatants (transfected or co-transfected as indicated) were mixed with recombinant proteins (1 µg) incubated 2 h at 4 °C under gentle agitation. As control, a further tube containing the corresponding cytokine was incubated without receptor containing supernatant. 150 µl of protein A-agarose (Roche Diagnostics GmbH, Mannheim, Germany) was added, and the mixture was incubated at 4 °C for 2 h under gentle agitation. The samples were washed six times with PBS, and proteins were eluted by adding 50 µl of Laemmli buffer and incubated for 10 min at 95 °C. The resulting supernatants were subjected to Western blot analysis.

**Western Blotting**—For Western blotting, ~2 × 10^7 cells per experiment were washed three times with sterile PBS. The cells were distributed to 2-ml tubes and starved in FBS-free medium for 4–6 h (Ba/F3 cell lines) or overnight (HepG2 cells) at 37 °C and CO_2 saturation. Cells were stimulated with the indicated cytokines for 10 min followed by centrifugation at 4 °C and 2,000 rpm for 10 min. 150 µl of 5× Laemmli buffer (312 mM Tris-HCl, pH 6.8, 50% glycerol, 10% sodium dodecyl sulfate, 5% β-mercaptoethanol, and 0.13% bromphenol blue) were added to each tube, and cells were lysed by sonification (Sonopuls HD2200, Bandelin, Berlin, Germany) for 10 s and boiling at 95 °C for 10 min. Proteins were separated by SDS-PAGE and transferred to a PVDF membrane using a Trans-Blot SD semi-dry transfer cell (Bio-Rad). The membrane was blocked in 5% low fat milk in TBS-T (10 mM Tris-HCl, pH 7.6, 150 mM NaCl, and 1% Tween 20) and probed with the primary antibody in 1% low fat milk in TBS-T (STAT3-mAb) or 5% BSA (pSTAT3-mAb) at 4 °C overnight. The membranes were washed and incubated with the secondary peroxidase-conjugated antibody for 1 h before applying the ECL-plus peroxidase substrate (GE Healthcare). The Fluor ChemQ system (Cell Biosciences, Santa Clara, CA) was used for signal detection according to the manufacturer’s instructions. The membranes were stripped with stripping buffer (20 ml of 10% SDS, 12.5 ml of 0.5 M Tris-HCl, pH 6.8, 67.5 ml of ultra-pure water, 0.8 ml of β-mercaptoethanol), blocked again, and probed with another primary antibody. The STAT3 phosphorylation assays were reproduced three times with one representative experiment shown.

**RESULTS**

**Cloning, Bacterial Expression, Refolding, and Purification of CNTF and the CNTF Variants CV-1 to -5**—Binding of CNTF to its α-receptors CNTFR or IL-6R is mediated via site I of CNTF and the cytokine binding module of the α-receptors. CNTF has a higher affinity to the CNTFR than to the IL-6R (3). Also, CLC needs the CNTFR for signaling via gp130 and LIFR, but cannot bind to the IL-6R. CLC has, however, a lower efficiency to signal via CNTFR-gp130-LIFR complexes as compared with CNTF (16). Fig. 1A illustrates the binding site I of CLC and CNTF, respectively. In this study, we aimed to generate a CNTFR-selective CNTF variant without influencing the affinity of CNTF to the CNTFR-gp130-LIFR complex. In the past, successful transfer of the binding site III of CNTF to IL-6 led to the chimeric IL-6 variant IC-7, which signals via the artificial IL-6R-gp130-LIFR complex (9). Here, this strategy was also applied to site I of CNTF. We generated potential CNTFR-selective CNTF variants by transfer of amino acid residues of site I from CLC to CNTF (Fig. 1A). Alternatively, using molecular modeling of CLC-CNTFR and CNTF-CNTFR complexes, we predicted that the amino acid Glu-26 in CLC might be involved in CNTFR selectivity because it is in close contact to the CNTFR (Fig. 1B). The respective amino acid in CNTF was identified to be Arg28.

As a starting point, we generated a shortened CNTF variant to improve recombinant CNTF protein expression in E. coli and to support the refolding efficiency from CNTF inclusion bodies. Therefore, the cDNA coding for CNTF was modified to code for a N-terminal deletion of the amino acids 1–14 and a C-terminal deletion of the amino acids 186–200 (17). Moreover, two point mutations coding for the amino acid exchanges C17A and Q63R were introduced (Fig. 1C). With the exception of the C-terminal deletion, all other modifications were described previously for the CNTF variant Axokine. Axokine was shown to have an improved biological activity as compared
A sequence coding for a C-terminal c-Myc and His tag was introduced in all cytokine-encoding cDNAs, including CNTF, CLC, IL-6, LIF, and IC-7. The cDNA coding for CNTF served as a platform for the transfer of amino acids from CLC to CNTF in CV-1 to CV5. CV-1 contained the single amino acid substitution R28E, located in close proximity to site I, CV-2 contained the extended site Ia (CNTF-His41-Leu67 to CLC-Gly41-Leu72) and CV-3 the minimal site Ia (CNTF-Ala59-Leu67 to CLC-Ala62-Leu72), CV-4 contained the site Ib (CNTF-Thr169-Gly185 to CLC-Leu170-Pro186) and CV-5 contained all exchanges, the extended site Ia and site Ib and the amino acid substitution R28E (CNTF-His41-Leu67 to CLC-Gly41-Leu72/CNTF-Thr169-Gly185 to CLC-Leu170-Pro186/R28E) (Fig. 1C).

CLC, CNTF, CV-1, CV-2, CV-3, CV-4, CV-5, IL-6, and IC-7 were expressed in E. coli as inclusion bodies, refolded, and purified. Purification was completed by nickel-nitrilotriacetic acid affinity chromatography and size-exclusion chromatography. All cytokines were produced as monomers (Fig. 2A and supplemental Fig. 1) and correctly folded as verified by circular dichroism revealing the typical spectra of helical proteins (Fig. 2B and supplemental Fig. 1). Purity and identity of correctly
folded and monomeric CLC, CNTF, CV-1 to CV-5, IL-6, and IC-7 was shown by Coomassie staining of SDS-PAGE gels and Western blotting against the C-terminal cytokine Myc tag, respectively (Fig. 2, C and D, and supplemental Fig. 1, A–H).

The CNTF Variants (CV-1, CV-2, CV-3, CV-4, CV-5) Bind to CNTFR, but only CV-3 and CV-4 Bind to IL-6R—First, we tested whether CNTF, CLC, IL-6, and the CNTF variants CV-1 to CV-5 were able to bind to CNTFR or IL-6R by co-precipitation. To this end, cDNAs coding for sCNTFR and sIL-6R C-terminally fused to an Fc part of an IgG1 antibody were subcloned and transiently transfected into COS-7 cells. sIL-6R-Fc or sCNTFR-Fc conditioned cell culture supernatants were used for Protein A-mediated co-precipitation of recombinant CNTF, CLC, IL-6, and CV-1 to CV-5. Precipitated cytokines were detected by Western blotting using a mAb against the C-terminal c-Myc tag. Non-precipitated input served as positive control, precipitation without sCNTFR-Fc or sIL-6R-Fc as negative control. As expected, co-precipitation of CLC and CNTF but not of IL-6 was mediated by sCNTFR-Fc or sIL-6R-Fc as negative control. As expected, co-precipitation of CLC and CNTF but not of IL-6 was mediated by sCNTFR-Fc or sIL-6R-Fc as negative control. As expected, co-precipitation of CLC and CNTF but not of IL-6 was mediated by sCNTFR-Fc or sIL-6R-Fc as negative control. As expected, co-precipitation of CLC and CNTF but not of IL-6 was mediated by sCNTFR-Fc or sIL-6R-Fc as negative control. As expected, co-precipitation of CLC and CNTF but not of IL-6 was mediated by sCNTFR-Fc or sIL-6R-Fc as negative control. As expected, co-precipitation of CLC and CNTF but not of IL-6 was mediated by sCNTFR-Fc or sIL-6R-Fc as negative control. As expected, co-precipitation of CLC and CNTF but not of IL-6 was mediated by sCNTFR-Fc or sIL-6R-Fc as negative control. As expected, co-precipitation of CLC and CNTF but not of IL-6 was mediated by sCNTFR-Fc or sIL-6R-Fc as negative control.

Importantly, all CNTF variants CV-1, CV-2, CV-3, CV-4, and CV-5 were also precipitated by sCNTFR-Fc, indicating that CV-1, CV-2, CV-3, CV-4, and CV-5 were able to form heterodimeric complexes with the CNTFR (Fig. 3A). As expected, co-precipitation of IL-6 and CNTF but not of CLC was also mediated by sIL-6R-Fc (Fig. 3B). Interestingly, only the CNTF-variant CV-3 and CV-4 were co-precipitated by sIL-6R-Fc, indicating that the amino acid exchange(s) in CV-1, CV-2 and CV-5 were sufficient to abrogate interaction with the IL-6R (Fig. 3B). We concluded that CV-1, CV-2 and CV-5 have CNTFR-selective binding properties.

CV-1, -4, and -5 Have Lost the Ability to Induce Signaling via gp130-LIFR-IL-6R—To test CV-1 to CV-5 in a biological system, we generated Ba/F3 cells expressing IL-6R and gp130, or IL-6R, gp130, and LIFR. Proliferation of Ba/F3-IL-6R-gp130 cells is dependent on IL-6 via an IL-6R-gp130 receptor complex. As expected, only IL-6 but not LIF, CNTF and CV-1 to -5 was able to induce cellular proliferation of Ba/F3-IL-6R-gp130 cells.
After stable transfection of Ba/F3 cells with human gp130, LIFR, and IL-6R (referred to as Ba/F3-IL-6R-gp130-LIFR cells), proliferation of these cells was dependent on CNTF, LIF, and IC-7 via the IL-6R/H18528 gp130/H18528 LIFR receptor complex. As a control, CLC was not able to induce cellular proliferation of Ba/F3-IL-6R-gp130-LIFR cells because CLC specifically signals via a CNTFR/H18528 gp130/H18528 LIFR complex. As described previously, CNTF-induced proliferation of Ba/F3-IL-6R-gp130-LIFR cells was ~100-fold lower compared with IL-6-induced cellular proliferation (3). The CNTF-variants CV-1, -4, and -5 completely failed to induce cellular proliferation of Ba/F3-IL-6R-gp130-LIFR cells, whereas CV-3-induced proliferation was induced by 100–1000 ng/ml and was stronger as compared with CNTF. Surprisingly, 1000 ng of CV-2/ml induced proliferation of Ba/F3-IL-6R-gp130-LIFR cells (Fig. 4B).

HepG2 cells are responsive to CNTF via the CNTFR/H18528 gp130/H18528 LIFR complex (3, 19). Therefore, we analyzed STAT3 phosphorylation in HepG2 cells by Western blotting comparing the bioactivities of CNTF, CLC, IL-6, LIF, IC-7, and CV-1 to CV-5 (10 and 100 ng/ml).
IL-6, IC-7, and CV-1 to CV-5. As expected, LIF, IL-6, and IC-7 but not CLC induced STAT3 phosphorylation in HepG2 cells at 10 and 100 ng/ml, whereas only 100 but not 10 ng/ml were sufficient to induce STAT3 phosphorylation by CNTF (Fig. 4C). Of note, the lower concentration of CV-3 (10 ng/ml) induced STAT3 phosphorylation, supporting the notion that CV-3 is more efficient than CNTF to induce signaling via IL-6R(gp130)/LIFR complexes. In addition, at higher concentrations of CV-1 to CV-5 (1000 ng/ml), only CV-3 induced detectable STAT3 phosphorylation in HepG2 cells (Fig. 4D). Our findings showed that the three CNTF variants CV-1, CV-4, and CV-5 completely lost their ability to signal via the IL-6Rgp130/LIFR complex.

The CNTF Variant CV-1 Signals via the CNTFR/gp130/LIFR Complex with Equal Efficacy as Compared with CNTF—Next, we generated Ba/F3 cells expressing gp130/LIFR and CNTFR/gp130/LIFR. After stable transfection with cDNAs coding for human gp130 and LIFR (referred to as Ba/F3-gp130-LIFR cells), proliferation of Ba/F3-gp130-LIFR cells was dependent on IL-6 trans-signaling (Hyper-IL-6; IL-6/sIL-6R fusion protein) via a gp130 homodimer or on LIF via a gp130/LIFR heterodimer. As expected, CLC, CNTF, and CV1–5 were not able to induce cellular proliferation of Ba/F3-gp130-LIFR cells (Fig. 5A), again indicating that the Q63R in CNTF did not lead to activation of gp130 and LIFR without CNTFR. After stable transfection of Ba/F3 cells with human CNTFR/gp130/LIFR cDNAs (referred to as Ba/F3-CNTFR-gp130-LIFR cells), dose-dependent proliferation of Ba/F3-CNTFR-gp130-LIFR cells was also dependent on CNTF or CLC via a CNTFRgp130/LIFR receptor complex (Fig. 5B). To compare the biological activity of the CNTF variants CV-1 to CV-5, dose-dependent cytokine-induced proliferation of Ba/F3-CNTFR-gp130-LIFR cells was performed. Although all CNTF variants were precipitated with sCNTFR-Fc, the CNTF variants differ in their potency to induce cellular proliferation by a factor of 100 (Fig. 5C). Whereas the biological activity of CV-1 and CV-3 was comparable with CNTF, CV-2 and especially CV-4 and CV-5 revealed ~10- to 100-fold reduced biological activity as compared with CNTF (Fig. 5C). Furthermore, we also verified that CNTF, CV1, and CV5 induced STAT3 phosphorylation in Ba/F3-CNTFR-gp130-LIFR cells (Fig. 5D). Our data demonstrated that only CV-1 fulfilled the initial requirements, namely CNTFR selectivity and high biological activity.

DISCUSSION

CNTF prevents degeneration of axotomized peripheral motor neurons and retrograde cell death of neurons in thalamic.
nuclei after dissection of intracerebral neuronal circuits (20, 21). Intraprapetional implantation of a CNTF-producing cell line improved survival in a mouse model of motor neuropathy (22). In models of Huntington disease, CNTF has neuroprotective effects on striatal cells (23–25). CNTF also enhanced the survival of sensory, hippocampal, and cerebellar neurons (26–28) and increased survival of retinal photoreceptors in animal models of retinal degeneration (29–31). On denervated rat skeletal muscles, CNTF reduced denervation-induced atrophy and increased muscular strength (32, 33).

Moreover, CNTF is still unique in its ability to overcome leptin resistance and reduce food intake and body weight in obese, leptin-resistant humans and rodents. Importantly, weight loss was sustained after discontinuation of the drug (34). Because leptin resistance is still a major problem in obesity, CNTF was suggested as therapeutic principle to combat human obesity (35). It was hypothesized that CNTF has leptin-like actions in the hypothalamus. Activation of either leptin receptor or CNTFR in the hypothalamus leads to local STAT3 and mTOR activation (36–38). Both cytokines also reduce AMP-activated kinase phosphorylation (39, 40). Additionally, CNTF and leptin receptor expression is overlapping in the hypothalamus (41), including the paraventricular nucleus and arcuate nucleus (42–44). However, a recent report indicates that despite anatomical overlap of CNTFR and leptin receptor expression, CNTF and leptin act within distinct neuronal populations to elicit anorectic effects (45).

Here, we describe the development of the CNTFR-selective CNTF variant CV-1, which is characterized by the single amino acid exchange R28E from CLC to CNTF. Biological activity of CV-1 was comparable with CNTF on Ba/F3-CNTFR-gp130-LIFR cells, indicating that the affinity of CV-1 to CNTFR-gp130-LIFR complex was unaffected.

CV-4 contained the exchange of site Ib. Although this variant appeared to bind to the IL-6R, proliferation assays revealed that CV-4 was CNTFR-selective. Biological activity of CV-4 was, however, ~100-fold reduced as compared with CNTF and appeared to be similar to CLC. We have, however, no explanation why CV-4 binds to IL-6R but did not induce proliferation of Ba/F3-IL-6R/gp130/LIFR cells. CV-2 and CV-3 contained the extended or the minimal site Ia from CLC, respectively. Exchange of the minimal site Ia did not affect receptor selectivity and biological activity of CV-3 toward CNTFR/gp130/LIFR but, surprisingly, increased the biological activity toward IL-6R/gp130-LIFR complexes. Exchange of the extended site Ia in CV-2 abrogated interaction with IL-6R, but at the highest concentration applied, CV-2 still induced cytokine-dependent proliferation via the IL-6R/gp130-LIFR receptor complex, albeit no detectable STAT3 phosphorylation. Moreover, the biological activity toward CNTFR/gp130-LIFR complexes was reduced by a factor of 10 for CV-2. From these data, we concluded that simple combination of site Ia and site Ib would result in CNTFR-selectivity but with overall reduced biological activity toward CNTFR-gp130-LIFR complexes. Therefore, we decided to combine all site exchanges, including the amino acid substitution R28E in CV-5. Albeit CV-5 was CNTFR-selective, exchange of the complete site Ia and Ib from CLC to CNTF abolished the positive affinity effect of R28E observed in CV-1.

All CNTF variants were generated using the human CNTF cDNA. Murine CNTF has about 82% sequence homology with human CNTF. In CNTF, Arg28 and the surrounding amino acids are identical between mice and humans; therefore, we assume that either receptor selectivity will be transferrable to murine CNTF and/or CV-1 will maintain its receptor selectivity also on murine receptor complexes. This must, however, be studied before CV-1 will be tested in in vivo studies.

The CNTFR-selective CV-1 may be of particular interest in view of the therapeutic potential of CNTF in neurodegenerative diseases and obesity and may even allow the reinvestigation of the therapeutic effects of CNTF at higher dosages.

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