CNTF variants with increased biological potency and receptor selectivity define a functional site of receptor interaction

Isabella Saggio¹, Isabelle Gloaguen,
Giancarlo Poiana² and Ralph Laufer³

Istituto di Ricerche di Biologia Molecolare P. Angeletti (IRBM),
Via Pontina km 30.600, 00040 Pomezia, Rome and ²Department of
Cellular and Developmental Biology, University of Rome
'La Sapienza', 00185 Rome, Italy

¹Present address: CNRS, URA 1301, Génétique des Virus Oncogènes,
Institut Gustave Roussy, 94805 Villejuif Cedex, France

³Corresponding author

Human CNTF is a neurocytokine that elicits potent neurotrophic effects by activating a receptor complex composed of the ligand-specific α -receptor subunit (CNTFR α) and two signal transducing proteins, which together constitute a receptor for leukemia inhibitory factor (LIFR). At high concentrations, CNTF can also activate the LIFR and possibly other cross-reactive cytokine receptors in the absence of CNTFR α . To gain a better understanding of its structure–function relationships and to develop analogs with increased receptor specificity, the cytokine was submitted to affinity maturation using phage display technology. Variants with greatly increased CNTFR α affinity were selected from a phage-displayed library of CNTF variants carrying random amino acid substitutions in the putative D helix. Selected variants contained substitutions of the wild-type Gln167 residue, either alone or in combination with neighboring mutations. These results provide evidence for an important functional role of the mutagenized region in CNTFR α binding. Affinity enhancing mutations conferred to CNTF increased potency to trigger biological effects mediated by CNTFR α and enhanced neurotrophic activity on chicken ciliary neurons. In contrast, the same mutations did not potentiate the CNTFR α -independent receptor actions of CNTF. These CNTF analogs thus represent receptor-specific superagonists, which should help to elucidate the mechanisms underlying the pleiotropic actions of the neurocytokine.

Key words: affinity maturation/ciliary neurotrophic factor/ciliary neurotrophic factor receptor/cytokine/phage display

Introduction

Ciliary neurotrophic factor (CNTF) is a 23 kDa neurocytokine which is expressed in both the peripheral and central nervous system, beginning in the late embryonic period (reviewed by Manthorpe *et al.*, 1993; Sendtner *et al.*, 1994a). Initially identified by its ability to promote *in vitro* survival of embryonic chick parasympathetic neurons, CNTF was subsequently shown to exert potent growth-promoting and/or differentiating actions on a variety of

neuronal and glial cells, including motoneurons, sensory neurons, sympathetic neurons, hippocampal neurons and oligodendrocytes (reviewed by Manthorpe *et al.*, 1993; Sendtner *et al.*, 1994a). *In vivo* administration of CNTF prevents degeneration of chick spinal motoneurons during development (Oppenheim *et al.*, 1991), of axotomized rat facial motoneurons (Sendtner *et al.*, 1990) and of motoneurons in mutant *progressive motor neuronopathy* mice (Sendtner *et al.*, 1992). The neuroprotective effects of CNTF raised hopes for its possible clinical applications in the treatment of human neurodegenerative diseases (Lindsay *et al.*, 1994).

CNTF is a member of a family of proteins, including leukemia inhibitory factor (LIF), interleukin 6, oncostatin M and interleukin 11, characterized by the use of structurally related and partially shared receptor subunits (Kishimoto *et al.*, 1994; Stahl and Yancopoulos, 1994). Moreover, on the basis of structure predictions, CNTF was proposed to share with these proteins a four α -helix bundle topology similar to that of human growth hormone (hGH) (Bazan, 1991). Like other cytokines (Kishimoto *et al.*, 1994), CNTF exerts its biological effects through the activation of a multichain receptor complex (Stahl and Yancopoulos, 1994), consisting of a ligand-specific subunit (CNTFR α) and two structurally related transmembrane signal transducing proteins, gp130 and the LIF receptor- β (LIFR- β). Binding of CNTF to CNTFR α is thought to trigger heterodimerization of gp130 and LIFR- β , resulting in activation of a signal transduction cascade mediated by specific intracellular protein tyrosine kinases (Stahl and Yancopoulos, 1994).

Two striking features of this tripartite receptor complex are that its two transmembrane components constitute a functional receptor for LIF and that gp130 is present in all the receptors of the CNTF-related cytokine subfamily. This explains why these various cytokines often exert pleiotropic and functionally redundant actions (Kishimoto *et al.*, 1994). The high-affinity biological actions of CNTF depend on the ligand- and neuron-specific CNTFR α subunit (Davis *et al.*, 1993; Ip *et al.*, 1993). Yet, at high concentrations, CNTF can elicit CNTFR α -independent effects, presumably by activating cross-reactive cytokine receptors (Baumann *et al.*, 1993; Davis *et al.*, 1993; Nesbitt *et al.*, 1993; Gearing *et al.*, 1994). The molecular recognition mechanisms underlying the interaction of CNTF with its specific or shared receptor components are unknown.

The development of CNTF variants with enhanced receptor affinity and specificity is important for understanding the molecular basis of receptor activation, as well as for guiding the design of proteins with greater therapeutic potential. The technique of protein display on phage provides a powerful approach for structure–function analysis and the identification of protein variants with

altered receptor specificities (Smith and Scott, 1993; Clackson and Wells, 1994). We recently reported that human CNTF displayed on the surface of fd phage in fusion with the C-terminal domain of the phage coat protein pIII retained specific binding to human CNTFR α and full biological activity. The finding that CNTF-phage could be affinity purified on immobilized CNTFR α suggested that this system was applicable to the sorting of large repertoires of phage-displayed CNTF variants (Saggio *et al.*, 1995). In this paper we describe the construction and screening of a phage-displayed library of human CNTF mutants randomized in the putative D helix. Our results provide evidence for an important functional role of this region, which was previously hypothesized to contain receptor binding determinants. Furthermore, affinity selection led to the isolation of CNTF variants with greatly enhanced CNTFR α specificity and biological potency.

Results

Construction of a phage-displayed library of CNTF variants

By analogy with hGH, the prototype member of the α -helical cytokine superfamily, the putative D helix of CNTF has been proposed to contain determinants of CNTFR α binding (Bazan, 1991). According to structural predictions (Bazan, 1991), the D helix encompasses amino acid residues 157–180 of CNTF. To functionally analyze this region, random mutations were introduced within the 20 amino acid sequence 159–178, using codon-based mutagenesis (Glaser *et al.*, 1992; Cormack and Struhl, 1993). A degenerate oligonucleotide was synthesized with a mutant/wild-type codon ratio expected to yield a high proportion (61–75%) of sequences averaging between one and three mutated codons per oligonucleotide, with a relatively low proportion (4–12%) of wild-type sequences. For 20 randomized codons the number of individual sequences with one, two or three mutated XXG/C triplets is 640, 194 560 and 3.7×10^7 respectively. Hence, in a library containing 10^7 individual clones, ~70% of which harbor between one and three codon changes, all possible single and double amino acid substitutions, as well as a significant proportion of all triple substitutions, within a 20 amino acid region should be represented. The mutagenized oligonucleotide was used as a primer for PCR amplification of a wild-type human CNTF template and the amplified fragment was substituted for the corresponding wild-type sequence in the CNTF expression vector pHen Δ -CNTF (Saggio *et al.*, 1995). The resulting phagemid vector pHen Δ -MUT-CNTF contains the coding sequence for CNTF (randomized between residues 159 and 178) fused to that of the C-terminal domain of fd phage coat protein pIII. The presence of an amber stop codon between the CNTF and pIII sequences allows the production of either CNTF-displaying phage particles or soluble (periplasmic) cytokine in suppressor or non-suppressor strains of *Escherichia coli* respectively (Saggio *et al.*, 1994, 1995).

A library of CNTF mutants was generated by transformation of *E. coli* TG1 with pHen Δ -MUT-CNTF, yielding $\sim 10^7$ independent transformants. Enzymatic restriction analysis revealed that 14 of 16 clones harbored a CNTF insert of the expected size (data not shown).

DNA sequence analysis of 11 clones revealed that two had deletions within the mutagenized region, one had the wild-type sequence, and eight harbored between one and three mutated codons (Table I). The observed codon changes and resulting amino acid substitutions appeared to be random, with no discernible clustering. Even though the number of analyzed sequences was too small to draw statistically significant conclusions about the representativeness of the library, the observed frequency (8/11) of clones with one to three codon substitutions was in accordance with expectation.

The bacterial library was superinfected with M13KO7 helper phage to produce a phage-displayed library of CNTF mutants. The identity and integrity of the mutagenized CNTF construct was confirmed by sequence analysis of the phage library DNA corresponding to the entire CNTF coding sequence, cloning sites and adjacent leader sequence. The sequence of the pooled library inserts was found to be identical to that of wild-type CNTF even in the randomized region, in agreement with the expected codon mutation frequency of only 10–15% at any given position.

Affinity selection of CNTFR α binders

To select receptor-specific CNTF variants, the phage library was submitted to three consecutive rounds of affinity purification using immobilized baculovirus-expressed myc-tagged human CNTFR α (s-CNTFR α ; Saggio *et al.*, 1995). Individual selectants were analyzed by DNA sequencing. In parallel, the corresponding secreted forms of CNTF variants were produced in the periplasm of a non-suppressor strain of *E. coli* and quantified by ELISA using an antibody directed against a CNTF epitope located outside the mutagenized region (Rende *et al.*, 1992). There were no significant differences between the amounts of wild-type and mutant CNTF recovered from bacterial periplasm (see Materials and methods), indicating that the selected mutations did not affect protein expression or secretion. The secreted proteins were tested for CNTF receptor binding by assessing their ability to compete with biotinylated CNTF for binding to s-CNTFR α (Saggio *et al.*, 1994). The proportion of active variants dramatically increased from the starting library through the enrichment cycles. Of 11 clones from the unselected library (see Table I), only the one with the wild-type sequence encoded a protein with detectable receptor binding activity (data not shown). In contrast, 5/20, 20/20 and 32/36 analogs from the first, second and third rounds of selection respectively were found to be at least equipotent with CNTF (see below).

To characterize the receptor binding characteristics of the selected variants, IC₅₀ values were determined from complete dose–inhibition curves and compared with that of wild-type CNTF, yielding an estimate of relative binding affinities. These data, together with the corresponding amino acid changes, are summarized in Table II. Among 56 proteins encoded by phagemid clones derived from the second and third rounds of affinity selection, seven had no amino acid substitutions within the randomized 159–178 sequence and, consistently, were equipotent with CNTF in binding to s-CNTFR α . Four clones had deletions and gave rise to either inactive or poorly expressed proteins (data not shown). The other selected isolates encoded 21

Table I. Sequences of random clones from the CNTF mutant library

Clone	Residue position																			No. of mutations	
	159	160	161	162	163	164	165	166	167	168	169	170	171	172	173	174	175	176	177		178
Wild-type	cta	aag	gtg	ctg	cag	gag	ctt	tca	cag	tgg	aca	gta	agg	tcc	atc	cat	gac	ctt	cgt	ttc	
Lib1	L	K	V	L	Q	E	L	S	Q	W	T	V	R	S	I	H	D	L	R	F	1
	-	-	-	-	-	-	-	-	-	-	-	-	-	-	tag	-	-	-	-	-	
Lib2	-	-	-	agc	-	-	ggg	-	-	-	-	-	-	-	-	-	-	-	-	-	2
	-	-	-	S	-	-	G	-	-	-	-	-	-	-	-	-	-	-	-	-	
Lib3	-	-	-	-	-	ctg	-	-	-	-	-	-	-	-	-	-	-	aac	-	acc	3
	-	-	-	-	-	L	-	-	-	-	-	-	-	-	-	-	-	N	-	T	
Lib4	-	-	-	-	-	-	-	-	-	ctc	-	-	-	-	-	-	ggc	ccc	-	-	3
	-	-	-	-	-	-	-	-	-	L	-	-	-	-	-	-	G	P	-	-	
Lib5	-	-	-	-	gtc	-	-	-	-	-	-	-	-	ggg	-	-	-	-	-	-	2
	-	-	-	-	V	-	-	-	-	-	-	-	-	G	-	-	-	-	-	-	
Lib6	-	ccc	-	-	atc	-	-	-	-	-	-	-	-	-	-	-	-	-	aac	-	3
	-	P	-	-	I	-	-	-	-	-	-	-	-	-	-	-	-	-	N	-	
Lib7	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	
	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	
Lib8	-	-	-	ccg	-	-	aac	-	-	-	-	-	-	-	-	-	-	-	-	-	2
	-	-	-	P	-	-	N	-	-	-	-	-	-	-	-	-	-	-	-	-	
Lib9	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	0

The DNA and protein sequences corresponding to the randomized region (residues 159–178) of wild-type CNTF are shown. For nine random clones (Lib1–Lib9) from the CNTF mutant library, only the codons and amino acid residues that differ from those of the wild-type are shown. The asterisk denotes a glutamine residue synthesized from an amber stop codon (tag) in a suppressor strain of *E.coli*.

Table II. Sequences and relative binding activities of selected CNTF variants

Residue position																				No. of isolates	Relative binding (CNTF = 1) ^a	
159	160	161	162	163	164	165	166	167	168	169	170	171	172	173	174	175	176	177	178			
L	K	V	L	Q	E	L	S	Q	W	T	V	R	S	I	H	D	L	R	F	(WT)	7	1.1 ± 0.3
-	-	-	-	-	-	-	-	T	-	-	-	-	-	-	-	-	-	-	-		3	11.8 ± 0.7
-	Q	-	-	-	-	-	-	T	-	-	-	-	-	-	-	-	-	-	-		1	3.1 ± 1.0
-	-	-	-	-	-	-	-	Y	-	-	-	-	-	-	-	-	-	-	-		1	9.6 ± 2.6
-	-	-	-	-	-	-	D	H	-	-	-	-	-	-	-	-	-	-	-		3	22.8 ± 3.5
-	-	-	-	S	-	-	-	H	-	-	-	-	-	-	-	-	-	-	-		1	4.1 ± 1.1
-	-	-	-	-	-	-	-	A	-	-	-	-	-	-	-	-	-	-	-		17	9.0 ± 0.7
-	-	-	-	-	-	-	A	A	-	-	-	-	-	-	-	-	-	-	-		1	8.1 ± 2.8
-	-	-	-	-	-	-	G	A	-	-	-	-	-	-	-	-	-	-	-		1	7.5 ± 2.2
-	-	-	-	-	-	-	N	A	-	-	-	-	-	-	-	-	-	-	-		1	12.4 ± 1.2
-	-	-	-	-	-	-	H	A	-	-	-	-	-	-	-	-	-	-	-		1	8.8 ± 2.6
-	-	-	-	-	-	-	D	A	-	-	-	-	-	-	-	-	-	-	-		4	13.5 ± 1.7
-	-	L	-	-	-	-	-	A	-	-	-	-	-	-	-	-	-	-	-		1	8.8 ± 0.4
-	Q	-	-	-	-	-	-	A	-	-	-	-	-	-	-	-	-	-	-		1	11.7 ± 3.2
-	-	-	-	-	-	-	-	A	-	-	-	-	-	-	A	-	-	-	-		1	3.6 ± 0.8
-	-	-	-	-	-	-	-	A	-	-	-	-	-	-	-	-	-	L	-		2	11.7 ± 3.3
-	-	-	-	-	-	-	-	A	-	S	-	-	-	-	-	-	-	-	-		1	6.9 ± 1.3
-	-	-	-	-	-	-	-	A	-	L	-	-	-	-	-	-	-	-	-		1	9.6 ± 2.2
-	-	-	-	-	-	-	-	A	-	L	-	-	-	-	-	-	L	-	I		1	8.4 ± 0.4
-	-	-	-	-	-	-	D	A	-	L	-	-	-	-	-	-	-	-	-		1	21.0 ± 1.6
-	-	-	-	-	-	-	D	A	-	-	-	-	-	-	-	-	-	F	-		1	13.1 ± 2.0
-	-	-	-	-	-	-	-	-	-	-	R	-	-	-	A	-	-	-	-		1	3.3 ± 0.4

The amino acid substitutions found at each randomized position (residues 159–178) are shown for 52 clones isolated after two or three cycles of affinity selection. The corresponding wild-type (WT) sequence is shown on top. Secreted forms of the variants were produced in the periplasmic space of *E.coli* HB2151 and quantified by ELISA. The S166D/Q167A/T169L CNTF variant was quantified by Western blotting analysis (Saggio *et al.*, 1994). Proteins were tested for their ability to compete with biotinylated CNTF for s-CNTFR α binding in the presence of s-gp130.

^aResults are expressed as the ratio between IC₅₀ values of standard secreted CNTF and the tested variant. The data represent mean values $\pm$ SEM from experiments performed with all different isolates of the same sequence or from at least two experiments for single isolates.

different sequences, each carrying between one and three mutations and displaying from 3- to 20-fold lower IC₅₀ values than the wild-type cytokine (Table II). Strikingly, the Q167 residue was found to be substituted with either A, H, Y or T in 20 out of these 21 variants. In fact, the most frequently isolated variant (17 isolates) carried a Q167A substitution. This protein, heretoforth designated

MUT-A, inhibited s-CNTFR α binding of biotinylated CNTF with a 9-fold lower IC₅₀ than CNTF. The only variant without a Q167 substitution was V170R/H174A, which displayed 3-fold higher binding activity than the wild-type. This effect is likely to be conferred by the V170R substitution, since H174A caused a 3-fold reduction in activity of the Q167A mutant (see Table II).

Fig. 1. CNTFR α binding competition between biotinylated CNTF and purified CNTF or variants. Experiments were performed in the presence (A) or absence (B) of s-gp130. ●, CNTF; Δ , MUT-A; $\square$, MUT-DH; $\circ$, MUT-DAL. Each point is the mean $\pm$ SD from two separate experiments, each carried out in duplicate. IC₅₀ values (ng/ml) were calculated by non-linear least square fitting: CNTF, 332 ± 20 and 678 ± 47 ; MUT-A, 19.2 ± 0.6 and 21.3 ± 1.2 ; MUT-DH, 10.8 ± 0.5 and 10.5 ± 0.9 ; MUT-DAL, 12.6 ± 0.2 and 10.4 ± 0.9 (in the presence and absence of s-gp130 respectively).

Additional mutations were found at the adjacent residue 166, as well as at positions 160, 161, 163, 169, 174, 177 and 178. Most of these did not greatly alter receptor binding. The effect of some substitutions depended on the mutational background. For instance, K160Q caused a 4-fold reduction of activity in the context of a Q167T substitution, but was without effect in a Q167A background (see Table II). Neither S166D nor T169L enhanced the activity of Q167A, yet these mutations were found in the two most potent variants, S166D/Q167H, designated MUT-DH, and S166D/Q167A/T169L, designated MUT-DAL, which displayed ~ 20 -fold lower IC₅₀ values than CNTF (Table II).

To validate these results, the coding sequences for MUT-A, MUT-DH and MUT-DAL were subcloned into a bacterial expression vector that allows high level protein expression and milligram amounts of the analogs were produced and purified. Figure 1A depicts the ability of the purified proteins to compete with biotinylated CNTF for binding to s-CNTFR α . The IC₅₀ values for MUT-A, MUT-DH and MUT-DAL (19, 11 and 13 ng/ml) were 17-, 31- and 26-fold lower respectively than that of the wild-type molecule (330 ng/ml). These results are in

reasonable agreement with those determined using crude protein preparations (see Table II) and confirm that affinity selection led to the isolation of CNTF derivatives with significantly improved receptor interaction.

The binding assays described above were carried out in the presence of the extracellular domain of human gp130 (s-gp130), which was previously reported to stabilize receptor binding of biotinylated CNTF (Saggio *et al.*, 1994). Since s-gp130 was also present during the library enrichment, we wished to determine whether it had any effect on the receptor interaction of selected variants. In the absence of s-gp130, MUT-A, MUT-DH and MUT-DAL competed for s-CNTFR α binding 32-, 65- and 65-fold more potently respectively than CNTF (Figure 1B). These results demonstrate that the tight binding of selected variants is due to increased affinity for the α -receptor component, rather than to improved interaction with the β -receptor subunit gp130.

Biological activity and receptor selectivity of selected CNTF variants

The biological activities of selected variants were tested in two human cell lines, TF-1 (erythroleukemia) and HepG2 (hepatoma), which express functional LIF receptors, but not CNTFR α (Baumann *et al.*, 1993; Davis *et al.*, 1993). Cells with these characteristics can be stimulated by high concentrations of CNTF, presumably because of a low affinity interaction of the cytokine with the LIFR- β /gp130 complex (Baumann *et al.*, 1993; Davis *et al.*, 1993; Gearing *et al.*, 1994). In the presence of exogenously added soluble CNTFR α , LIF receptor-bearing cells become responsive to low concentrations of CNTF, due to the formation of a high-affinity CNTF receptor complex composed of CNTFR α , LIFR- β and gp130 (Taga *et al.*, 1992; Baumann *et al.*, 1993; Davis *et al.*, 1993; Gearing *et al.*, 1994; Panayotatos *et al.*, 1994; Zhang *et al.*, 1994). This system therefore offers the possibility of discriminating, using the same cell type, between CNTFR α -specific and non-specific biological effects of CNTF variants.

HepG2 cells respond to CNTF by increasing the production of acute phase proteins (Schooltink *et al.*, 1992; Baumann *et al.*, 1993). To test whether this effect could be potentiated by the α -receptor, we determined the cytokine concentrations needed to evoke half maximal stimulation of haptoglobin production in HepG2 cells (EC₅₀) as a function of different concentrations of exogenously added s-CNTFR α . Addition of s-CNTFR α led to a dose-dependent reduction in the EC₅₀ values for CNTF and MUT-DH (Figure 2A). The extent of the potency shift depends on the cytokine's responsiveness to the α -receptor component (Panayotatos *et al.*, 1994). For CNTF the EC₅₀ was reduced ~ 10 -fold by the addition of 4 ng/ml s-CNTFR α (from 116 ± 32 to 10 ± 1 ng/ml). The potency shift for MUT-DH was more pronounced (from 143 ± 49 to 0.6 ± 0.06 ng/ml, ~ 200 -fold), reflecting the improved α -receptor interaction of this variant (Figure 2A).

To compare the relative biological activities of selected variants on both α -receptor-dependent and α -receptor-independent responses of HepG2 cells, experiments were carried out either in the absence or presence of a fixed concentration (0.8 ng/ml) of s-CNTFR α . As shown in Figure 2B, all tested analogs evoked dose-dependent and

Fig. 2. Stimulation of haptoglobin production in HepG2 cells by CNTF or CNTF variants. (A) Effect of added s-CNTFR α on the EC₅₀ values for CNTF (circles) or MUT-DH (squares). (B) Dose-response curves in the absence of s-CNTFR α . (C) Dose-response curves in the presence of 0.8 ng/ml s-CNTFR α . ●, CNTF; Δ, MUT-A; □, MUT-DH; ○, MUT-DAL. The data shown are from a typical experiment, carried out in duplicate culture wells. Average relative potencies (EC₅₀ CNTF/EC₅₀ variant) from two to three independent experiments were (mean $\pm$ SEM): MUT-A, 0.9 $\pm$ 0.5 and 19 $\pm$ 4; MUT-DH, 0.8 $\pm$ 0.2 and 20 $\pm$ 3; MUT-DAL, 0.4 $\pm$ 0.2 and 13 $\pm$ 1 (in the absence and presence of s-CNTFR α respectively).

saturable increases in the amount of haptoglobin secreted into the culture medium of HepG2 cells. In the absence of exogenous α -receptor, MUT-A, MUT-DH and MUT-DAL displayed similar potencies to wild-type CNTF (Figure 2B). This result shows that the CNTFR α -independent biological activity of CNTF is not potentiated by amino acid substitutions that enhance interaction with the α -receptor subunit. In contrast, the variants were 13- to 20-fold more potent than CNTF when tested in the presence of s-CNTFR α (Figure 2C). Thus the improved receptor binding activities of CNTF variants were paralleled by increases in their biological potency and receptor selectivity.

Similar results were obtained with the TF-1 erythroleukemia cell line. CNTF stimulates the survival of TF-1 cells maintained in the absence of other growth factors (Taga *et al.*, 1992; Davis *et al.*, 1993). Addition of s-CNTFR α led to 7- and 110-fold reductions in the EC₅₀ values for CNTF and MUT-DH respectively (Figure 3A). In the absence of α -receptor there were no significant differences between the potencies of CNTF, MUT-A, MUT-DH and MUT-DAL (Figure 3B). In contrast, the variants were 15- to 20-fold more active than CNTF when tested in the presence of 8 ng/ml s-CNTFR α (Figure 3C).

Neurotrophic activity of CNTF variants

To determine whether constitutively expressed neuronal CNTFR α was also sensitive to amino acid substitutions that enhance the affinity of CNTF for its soluble α -receptor, the neurotrophic effects of CNTF and variants were compared in the chick ciliary neuron bioassay (Manthorpe *et al.*, 1993; Sendtner *et al.*, 1994a). As depicted in Figure 4, a 20 h treatment of E8 chick ciliary ganglion neurons with CNTF led to a dose-dependent and saturable increase in the number of surviving cells, with an EC₅₀ of 0.5 ng/ml. MUT-A, MUT-DH and MUT-DAL behaved as full agonists in this system and displayed

4- to 8-fold higher neurotrophic activities than CNTF (Figure 4).

Discussion

In the present study phage display technology was used to screen and sort CNTF mutants on the basis of their affinity for CNTFR α . Affinity maturation led to the isolation of variants with selectively improved CNTFR α interaction. Apart from providing important insights into the structure-function relationships of CNTF, this approach allowed us to identify cytokine analogs with modified and potentially useful biological properties.

In vitro selection using phage-displayed protein libraries represents a powerful new strategy in protein engineering and drug discovery (Smith and Scott, 1993; Clackson and Wells, 1994). One of its limitations is that only four or five amino acid residues can be simultaneously randomized in a standard library, since the size of the library would otherwise exceed the limit imposed by DNA transfection efficiency (Lowman and Wells, 1993; Clackson and Wells, 1994). In the absence of any prior structure-function data on CNTF, it was necessary to sample a relatively large protein region. This was achieved using the method of codon randomization (Glaser *et al.*, 1992; Cormack and Struhl, 1993), which allowed the construction of a complex, yet highly compact, library of mutant CNTF molecules. The present approach provides a convenient alternative to selection strategies based on standard oligonucleotide-directed mutagenesis in cases where it is desirable to test the effects of a small number of random mutations within a large target region.

Phage display proved to be very efficient for the selection of high-affinity CNTF mutants. Inactive variants, which constituted the majority in the starting library, were virtually eliminated after only two rounds of binding enrichment, while 80% of selected variants exhibited

Fig. 3. Stimulation of TF-1 cell survival by CNTF or CNTF variants. (A) Effect of added s-CNTFR α on the EC₅₀ values for CNTF (circles) or MUT-DH (squares). (B) Dose-response curves in the absence of s-CNTFR α . (C) Dose-response curves in the presence of 8 ng/ml s-CNTFR α . ●, CNTF; Δ, MUT-A; □, MUT-DH; ○, MUT-DAL. The data shown are from a typical experiment, carried out in duplicate culture wells. Average relative potencies (EC₅₀ CNTF/EC₅₀ variant) from two to four independent experiments were (mean $\pm$ SEM): MUT-A, 1.8 $\pm$ 0.7 and 18 $\pm$ 4; MUT-DH, 0.7 $\pm$ 0.3 and 15 $\pm$ 2; MUT-DAL, 1.1 $\pm$ 0.4 and 19 $\pm$ 3 (in the absence and presence of s-CNTFR α respectively).

Fig. 4. Stimulation of survival of chick ciliary ganglion neurons by CNTF or CNTF variants. ●, CNTF; Δ, MUT-A; □, MUT-DH; ○, MUT-DAL. Each point is the mean $\pm$ SEM from six (CNTF, MUT-DH) or two (MUT-A, MUT-DAL) separate experiments. At saturating concentrations, CNTF caused a 5- to 10-fold increase in the number of viable cells, relative to untreated controls. To normalize for differences in absolute responses between different experiments, neurotrophic activity was calculated as a percentage of the maximal CNTF effect. EC₅₀ values (ng/ml) were calculated by non-linear least square fitting: CNTF, 0.50 $\pm$ 0.04; MUT-DH, 0.06 $\pm$ 0.01; MUT-A, 0.09 $\pm$ 0.02; MUT-DAL, 0.13 $\pm$ 0.02.

tighter interactions with CNTFR α than the wild-type molecule. The most striking outcome of the affinity selection was that there appeared to be only one solution for significantly improving the binding of CNTF, namely substitution of the Q167 residue. The most potent variants contained neighboring substitutions (S166D and T169L) which did not enhance affinity individually or within a different mutational background. The possibility to co-select such mutations constitutes one of the powerful features of affinity maturation from combinatorial libraries (Lowman and Wells, 1993; Clackson and Wells, 1994).

The most frequently isolated selectant had a single

Q167A substitution and possessed ~20-fold higher apparent affinity for the CNTFR α /gp130 complex than wild-type CNTF. The finding that receptor binding was also increased by changing Q167 into other non-homologous residues, such as threonine, histidine or tyrosine, indicates that the wild-type glutamine residue impairs receptor interaction. It is noteworthy that Q167 is also found in rat and mouse CNTF, as well as in growth promoting activity (GPA), the possible chicken homolog of CNTF (see Table III). Yet rabbit CNTF contains a histidine residue at that position and it will be interesting to determine the affinity of this protein for CNTFR α . The residue at position 167 of CNTF was previously proposed to be located within a structural determinant (D2) hypothesized to participate in receptor binding (Bazan, 1991). It is tempting to speculate that the Q167 residue is functionally analogous to the E174 residue adjacent to the corresponding D2 motif of hGH, whose substitution with alanine was found to enhance receptor binding because of unfavorable packing of the wild-type residue within the receptor binding interface (Wells et al., 1993).

It is interesting to note that most of the selected amino acid substitutions mapped on the hydrophilic face of the proposed amphipathic D helix of CNTF (Figure 5). The only mutations found on the hydrophobic face were either homologous (T169S) or hydrophobic (V161L and T169L) substitutions (Figure 5). This result highlights the importance of the hydrophobic region, which is likely to face the core of the protein. Mutations in buried positions might destabilize the protein by disrupting packing interactions. That the potentially exposed hydrophilic face of the D helix is functionally important is suggested by the affinity-enhancing effect of the neighboring S166D, Q167A and V170R substitutions and by the conservation of wild-type residues E164, R171 and D175 after binding selection. The potential importance of the residues conserved after binding selection is also supported by their strong species conservation (see Table III). However,

Table III. Comparison of the randomized amino acid sequence of human CNTF with those of rat, mouse and rabbit CNTF and chicken GPA

Clone	Residue position																			
	159	160	161	162	163	164	165	166	167	168	169	170	171	172	173	174	175	176	177	178
Human	L	K	V	L	Q	E	L	S	Q	W	T	V	R	S	I	H	D	L	R	F
Rat																				V
Mouse																				V
Rabbit									H											V
Chicken			R		R			A			A				V	R				Q

Only residues that differ from those of human CNTF are shown. For references see Sendtner *et al.* (1994a).

Fig. 5. Amino acid residues substituted in high-affinity CNTF variants (boxed) map on the hydrophilic face of the putative D helix. Residues 159–178 of CNTF, contained within the predicted D helix, are shown in helical wheel format to highlight the amphipathic pattern of residues (Bazan, 1991). The putative helical face I contains mainly hydrophobic amino acids (bold type), while face II contains charged or hydrophilic residues. The percent frequency of mutation at each position is indicated in parentheses. For more details see Table II.

because of the relatively small number of analyzed clones, the contribution of each individual residue remains to be verified by further site-directed mutagenesis.

Taken together, the present results provide the first experimental evidence for an important functional role of the putative D helix of CNTF (Bazan, 1991) in CNTFR α interaction. It has previously been reported that a specific amino acid substitution within the putative A-B loop of CNTF enhances CNTFR α binding affinity (Panayotatos *et al.*, 1993). Thus the specific receptor binding epitope probably involves amino acid residues from both the D helix and A-B loop, similarly to other members of the α -helical cytokine superfamily, such as hGH (Wells *et al.*, 1993), interleukin 2 (Zurawski *et al.*, 1993), oncostatin M (Kallestad *et al.*, 1991), interleukin 6 (Savino *et al.*, 1993) and LIF (Robinson *et al.*, 1994). It should be pointed out, however, that the present mutagenesis data do not constitute formal proof for the involvement of the putative D helix in receptor binding. Mutations in the CNTF molecule could cause conformational changes that indirectly affect the CNTFR α binding site and possibly also sites responsible for interaction with other receptor subunits. A precise understanding of how these mutations affect receptor interaction will have to await determination of the three-dimensional structure of the ligand–receptor complexes.

The most potent variants, MUT-A, MUT-DH and MUT-

DAL, bound 30- to 60-fold more tightly to CNTFR α than the wild-type cytokine. If mutant binding to the α -receptor does not impair subsequent interaction with the signal transducing receptor components, such proteins should be more potent than the wild-type in generating an activated cytokine–CNTFR α complex and are therefore expected to possess increased biological potencies. Indeed, increased CNTFR α affinities were paralleled by 20- to 30-fold tighter binding to the CNTFR α –gp130 complex and 15- to 20-fold increased potencies to trigger biological responses mediated by s-CNTFR α . These analogs thus represent ‘superagonists’ able to activate the functional interaction of the α -receptor with gp130 and LIFR- β at considerably lower concentrations than the natural ligand. Phage display strategies have been used to engineer potent enzyme inhibitors (Roberts *et al.*, 1992) and receptor antagonists (Clackson and Wells, 1994), but to our knowledge the selection of mutants with enhanced biological activities has not been reported to date.

Superagonist activity is not restricted to the soluble α -receptor subunit, since the mutants were 4- to 8-fold more potent than CNTF in promoting the survival of chick ciliary ganglion neurons, which constitutively express functional CNTF receptors. The extent of this potency increase is lower than that observed with s-CNTFR α , possibly due to a specificity difference between chicken and human receptors. Alternatively, membrane-bound CNTF receptors might be less sensitive than soluble receptors to changes in ligand affinity. In systems with high signal amplification, the wild-type cytokine acts at very low concentrations, which may be close to the EC₅₀ limit set by the receptor concentration (Van Rossum, 1966). In addition, agonist potency might be limited by the rate of ligand-mediated receptor endocytosis (Wiley and Cunningham, 1981). Receptor internalization (which might be faster for cellular versus soluble receptors) would thus tend to mask the effects of changes in receptor affinity. Interestingly, a difference between relative potencies on soluble versus membrane-bound receptors has also been reported for rat CNTF. Thus the latter has 8- to 30-fold higher CNTFR α affinity than human CNTF and 30-fold higher activity with s-CNTFR α , yet it is only 5-fold more active on chick ciliary neurons (Panayotatos *et al.*, 1993, 1994). However, other workers have reported a higher relative activity of rat CNTF on ciliary neurons (Sendtner *et al.*, 1994b). Thus additional studies are needed to determine precisely the relationship between the receptor affinity of CNTF variants and their biological activities on the physiologically relevant membrane receptor. Such studies would be facilitated by the availability of variants

with further improved receptor binding affinities. We are currently attempting to combine affinity-enhancing amino acid substitutions from different protein regions, which is expected to yield additive increases in receptor affinity (Lowman and Wells, 1993).

The finding of superagonist variants raises the question why affinity-enhancing mutations were not selected during evolution. One possibility is that during development a superactive neurotrophic factor might present the risk of stimulating the survival of cells that are normally programmed to die in the presence of limiting amounts of survival factor (Raff *et al.*, 1993). In addition, natural selection might favor cytokine residues that are compatible with binding to multiple receptors, rather than those which optimize binding to a particular receptor (Lowman and Wells, 1993).

Sorting CNTF variants on the basis of CNTFR α affinity resulted in a selective improvement of α -receptor interaction. The finding of CNTF analogs possessing increased biological activities in hepatoma and erythroleukemia cells in the presence, but not in the absence, of s-CNTFR α provides evidence for the interaction of CNTF with cross-reactive receptors displaying a different ligand specificity than the α -receptor subunit. The biological effects of CNTF on cells that do not express detectable amounts of CNTFR α mRNA have previously been proposed to be mediated by receptors for interleukin 6 (Nesbitt *et al.*, 1993) or LIF (Baumann *et al.*, 1993; Davis *et al.*, 1993). The latter hypothesis is supported by the observation that hematopoietic cells transfected with expression vectors encoding LIFR- β and gp130 proliferate in response to high concentrations of CNTF (Gearing *et al.*, 1994). Our present results indicate that the interaction of CNTF with LIF receptors involves a different site than that used for CNTFR α binding. The molecular localization of this epitope, which probably corresponds to the site at which CNTF contacts the LIFR- β chain within the context of the CNTF receptor complex (Stahl *et al.*, 1993), remains to be determined. An interesting possibility is that CNTF interacts with gp130 and LIFR- β at positions structurally equivalent to those of the two distinct gp130 binding sites of interleukin 6 (Paonessa *et al.*, 1995).

Cytokine analogs with improved biological activity and receptor selectivity constitute potentially useful tools for further studies of CNTF action. The neurotrophic actions of CNTF are mediated by its α -receptor subunit, which, in contrast to the widely distributed LIF receptor (Gearing, 1993), is expressed almost exclusively on the surface of neuronal cells (Ip *et al.*, 1993). In view of the short half-life and deleterious non-neuronal side effects of CNTF *in vivo* (Dittrich *et al.*, 1994; Henderson *et al.*, 1994), neuron-specific derivatives acting at low pharmacological doses could present an advantage for therapeutic application. Furthermore, receptor-selective analogs might help to dissect the molecular mechanisms underlying the pleiotropic actions of this cytokine.

Materials and methods

Construction of a phage-displayed library of CNTF mutants

To introduce random codon substitutions in the human CNTF DNA sequence encoding amino acid residues 159–178, a degenerate oligonucleotide was synthesized by codon-based mutagenesis (Glaser *et al.*,

1992; Cormack and Struhl, 1993). The oligonucleotide 5'-TGCTGGGAT-CCCAGTCTGATGAGAAGAAATGAAACCGAAGGTCATGGATGG-ACCTTACTGTCCACTGTGAAAGAGCTCCAGCACCCTTTAG-GCCCCACAGCTTCTCTCAAAGAGA-3' contains the antisense DNA sequence corresponding to the mutagenized region (nucleotides 475–534 of CNTF, in bold type), flanked by 5'- and 3'- constant regions. A *Bam*HI site used for cloning (see below) is indicated in italics. Oligonucleotide synthesis was performed according to Cormack and Struhl (1993). In brief, at the relevant step of the synthesis the resin was split into two portions that were repacked into mutant (10–15%) and wild-type (85–90%) columns. The wild-type column was subjected to three rounds of coupling with wild-type nucleotides, while on the mutant column a random G/CXX codon was synthesized, where X represents an equimolar mixture of the four nucleotide precursors. The two columns were then combined and the process repeated for each of the 20 codons to be mutated. The use of the G/CXX codon (for an antisense primer) ensures the (sense) synthesis of all 20 amino acids, while limiting the total number of triplets to 32. Only the amber stop codon (TAG) is encoded, which can be suppressed in *supE* strains of *E. coli*. For a mutant/wild-type coupling ratio of 10–15%, the expected frequency of sequences carrying no, one, two or three random codon substitutions was calculated (Derbyshire *et al.*, 1986) to be 4–12, 14–27, 23–29 and 19–24% respectively.

The mutated oligonucleotide was purified by high performance liquid chromatography and used as antisense primer in polymerase chain reaction (PCR) amplification of the 560 bp *Sall*–*Bam*HI fragment of the CNTF cDNA contained within the pHen Δ -CNTF phagemid vector (Saggio *et al.*, 1995). PCR was carried out for 25 cycles of 1 min at 94°C, 5 min at 60°C and 2 min at 72°C. The amplified fragment was digested with *Sall* and *Bam*HI and subcloned into the pHen Δ -CNTF vector from which the corresponding wild-type fragment had been excised. The resulting mutagenized phagemid pHen Δ -MUT-CNTF was electroporated into the *E. coli* strain TG1 and transformed cells were spread on 20 Petri dishes (15 cm diameter) containing LB agar, 1% (w/v) glucose and 50 μ g/ml ampicillin. Transformants ($\sim 10^7$ clonal clones) were pooled, resuspended in LB supplemented with 50 μ g/ml ampicillin and 15% (v/v) glycerol and stored at –70°C. DNA was extracted from some individual clones and analyzed by enzymatic restriction analysis. In addition, single bacterial clones were superinfected with M13K07 helper phage to produce recombinant phage (Saggio *et al.*, 1995), whose single-stranded DNA was extracted and analyzed by sequencing using the Sequenase protocol (USB).

To produce a phage-displayed library of CNTF mutants, an aliquot of the bacterial library was superinfected with M13K07 helper phage and recombinant phage particles were produced and purified by CsCl density gradient ultracentrifugation as described (Saggio *et al.*, 1995). Single-stranded DNA was extracted from the phage library and the sequence of the CNTF insert, as well as ~ 50 nt of the 5' and 3' flanking regions, was determined.

Affinity selection of receptor-specific CNTF variants

The library of phage-displayed CNTF variants was submitted to affinity selection on immobilized CNTFR α by a modification of the previously described micropanning procedure (Saggio *et al.*, 1995). In brief, s-CNTFR α (200 ng/ml), kindly provided by Dr G. Paonessa (IRBM), was immobilized on polystyrene plates (Falcon 1007) previously coated with a 20 μ g/ml solution of the anti-myc monoclonal antibody 9E10. Plates were incubated overnight at 4°C with 10^{12} phage particles/ml and 200 ng/ml s-gp130, kindly provided by Dr G. Paonessa. Following extensive washing, receptor-bound phage was eluted, amplified in *E. coli* TG1 and purified by CsCl gradient ultracentrifugation. The resulting phage preparation was cycled through two additional rounds of affinity selection, as described above. After each enrichment cycle, phagemid DNA, extracted from individual clones of *E. coli* TG1 infected with phage eluate, was analyzed by sequencing and used to produce secreted forms of CNTF variants in *E. coli* periplasm, as described below.

Expression and quantification of secreted forms of CNTF variants in bacterial periplasm

Phagemid DNA encoding CNTF or variant sequences was transformed into the *E. coli* non-suppressor strain HB2151 and selected against ampicillin. Bacterial periplasms were prepared from 50 ml bacterial cultures as described (Saggio *et al.*, 1994). The concentrations of CNTF variants in periplasmic extracts were determined by enzyme-linked immunosorbent assay (ELISA). The antibody used was affinity-purified IgG from rabbits immunized with a synthetic peptide corresponding to the rat CNTF (45–59) sequence (Rende *et al.*, 1992), which is located

outside the mutagenized CNTF region. The antibody was kindly provided by Dr M.Rende (University of Perugia, Italy). Standard CNTF or periplasmic extracts were incubated overnight at 4°C with 120 ng/ml biotinylated CNTF and 50 ng/ml antibody, in a final volume of 35 µl TBSMT [50 mM Tris-HCl, 150 mM NaCl, 5% (w/v) non-fat dry milk, 0.05% (v/v) Tween-20, pH 7.4]. The reaction mixtures were then transferred to individual wells of a polystyrene microtiter plate which had previously been coated with 0.25 µg affinity-purified goat antibodies against rabbit IgG. After 4 h at 4°C, wells were washed five times with TBST [50 mM Tris-HCl, 150 mM NaCl, 0.05% (v/v) Tween-20, pH 7.4], and 50 µl of a 1 µg/ml solution of alkaline phosphatase-conjugated avidin in TBST containing 0.5% (v/v) bovine serum albumin was added and left to incubate for 1 h at room temperature. Following additional washing (as above), alkaline phosphatase activity was determined at 37°C using 1 mg/ml *p*-nitrophenylphosphate in 1 M diethanolamine-HCl, pH 9.8. The absorbance at 405 nm was measured on a microplate reader. Competition curves obtained with purified CNTF were analyzed by non-linear regression and used to calculate the amount of CNTF variants in periplasmic extracts. Protein yields from 50 ml bacterial cultures ranged from 2.3 to 5.8 µg (3.8 ± 1.5 µg, mean $\pm$ SD, $n = 5$) for wild-type preparations and from 2.2 to 7.1 µg for mutant preparations.

Production and purification of CNTF variants from bacterial inclusion bodies

The coding sequences for selected CNTF variants contained within the pHenΔ-MUT-CNTF phagemid vector were amplified by PCR using the following sense and antisense primers: 5'-GTCACCATGGCTTTCACAGAGCATTCACCG-3' and 5'-TGACGCGGCCGCCCTACATTTTCTT-GTTGTTAGCAATATA-3' respectively. The sense primer contains an *NcoI* site (in italics), the ATG of which corresponds to the start codon of CNTF. The amplified sequence was subcloned as a *NcoI*-*Bam*HI fragment into the expression vector pRSET-CNTF (kindly provided by Dr S.Rose-John, University of Mainz, Germany) from which the corresponding wild-type CNTF fragment had been excised. The identity of all constructs was confirmed by DNA sequencing of the entire CNTF coding region. CNTF variants were expressed in *E.coli* BL21 (DE3) and purified from inclusion bodies as described (Saggio *et al.*, 1994), except that bacteria were grown in the presence of 1% (w/v) glucose before IPTG induction and protease inhibitors (1 mM phenylmethylsulfonyl fluoride and 10 µg/ml leupeptin) were included in the extraction buffers. Protein concentrations were determined by the BioRad protein assay, using as standard CNTF whose concentration was determined by amino acid analysis. Protein yields ranged from 10 to 20 mg/l bacterial culture, except for S166D/Q167A/T169L CNTF, whose yield was ~20-fold lower. All recombinant proteins were >90% pure, as determined by SDS-PAGE.

CNTFRα binding assay

The ability of secreted forms of CNTF variants to compete with biotinylated CNTF for binding to s-CNTFRα in the presence of s-gp130 was determined as previously described (Saggio *et al.*, 1994). For experiments with variants purified from inclusion bodies, the concentration of s-CNTFRα was lowered (using 2 ng of receptor for immobilization and a final reaction volume of 250 µl) in order to prevent ligand depletion. Under these conditions IC₅₀ values were not limited by receptor concentration. For experiments performed in the absence of s-gp130, binding reactions were carried out for 20 h at 4°C. IC₅₀ values were determined by non-linear regression from at least six different concentration points using the four-parameter logistic model (Rodbard and Frazier, 1975).

TF-1 cell survival bioassay

TF-1 human erythroleukemia cells (Kitamura *et al.*, 1989), kindly provided by Dr S.Alemà (CNR, Rome), were cloned by limiting dilution and a clone that exhibited low background survival in the absence of added growth factor was isolated and used for all further studies. For survival assays, cells were washed three times with RPMI 1640 and seeded at a density of 25 000 cells/well in 24-well tissue culture plates containing 0.5 ml culture medium (RPMI 1640, 3% horse serum, 2 mM glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin). Cells were treated for 3 days with different concentrations of purified wild-type or mutant CNTF, pulse-labeled for 3 h with 0.5 µCi/ml [methyl-³H]thymidine and harvested on glass fibre filters (96-well Packard GF/C Unifilter plates). Filters were washed and cell-associated radioactivity was determined in a Packard TopCount scintillation counter.

Assay of haptoglobin secretion from HepG2 cells

Confluent monolayers of HepG2 human hepatoma cells in 24-well tissue culture plates were placed in serum-free minimal essential medium containing 1 µM dexamethasone (Baumann *et al.*, 1993) and treated for 24 h with purified CNTF or variants. The amount of haptoglobin secreted into the culture medium was determined by ELISA as follows. Polystyrene 96-well microtiter plates were coated overnight at 4°C with 0.1% (v/v) delipidized goat antiserum against human haptoglobin (Sigma) in 100 µl 50 mM sodium carbonate buffer, pH 9.5. The following incubations were performed at room temperature and wells were washed six times with TBST after each incubation step: (i) 2 h with 250 µl TBSMT (blocking); (ii) 1 h with 50 µl TBSMT containing human haptoglobin standards or diluted HepG2 culture medium; (iii) 1 h with 50 µl TBSMT containing 0.1% (v/v) rabbit antiserum against human haptoglobin (Sigma); (iv) 1 h with 50 µl TBSMT containing 0.6 µg/ml alkaline phosphatase-conjugated donkey antibody against rabbit IgG (Pierce). Alkaline phosphatase activity was then determined as described for the CNTF ELISA. Standard dose-response curves were analyzed by non-linear regression and used to calculate the amount of haptoglobin in test samples.

Assay for neurotrophic activity

Survival of E8 chick embryo ciliary ganglion neurons was assayed essentially as described by Manthorpe and Varon (1989). Briefly, neurons were seeded at a density of ~3000 cells/well in laminin- and poly-D-lysine-coated 96-well tissue culture plates containing 100 µl culture medium (Dulbecco's modified Eagle's medium, 10% horse serum). Following a 20 h treatment with different concentrations of purified CNTF or variants, neurons were incubated for 4 h with 0.5 mg/ml of the vital dye MTT. Culture media were then removed and the formazan product was dissolved in 100 µl 20% (w/v) sodium dodecylsulfate in 50% (v/v) dimethylformamide, pH 4.7. Absorbances at 570 nm were determined on a microplate reader, using a reference filter of 690 nm.

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