Recombinant Human CNTF Receptor α: Production, Binding Stoichiometry, and Characterization of Its Activity as a Diffusible Factor

N. Panayotatos,* D. Everdeen, A. Liten, R. Somogyi, and A. Acheson

REGENERON Pharmaceuticals Inc., 777 Old Saw Mill River Road, Tarrytown, New York 10591-6707

Received December 20, 1993; Revised Manuscript Received March 4, 1994®

ABSTRACT: The primary ligand-binding protein (CNTFRα) of the multicomponent receptor for ciliary neurotrophic factor was produced in *Escherichia coli.* Using novel applications of size-exclusion chromatography and a protein gel-shift assay, we obtained quantitative separation of correctly refolded protein, as well as analytical monitoring of the refolding process and ligand binding. By these and other methods, we determined a 1:1 stoichiometry for the receptor–ligand complex. To investigate the proposed activity and mechanism of soluble CNTFRα as a diffusible factor, we studied the response of TF-1 cells which lack CNTFRα to various CNTF ligands and the stimulation of this response by sCNTFRα. The results show that sCNTFRα combines with CNTF and mediates cell survival with the same relative ligand specificity and relative affinity as the cell-surface form. Thus, soluble receptor can reconstitute on a cell surface active complexes that are analogous to the native complexes. Moreover, both the relative ligand potency in the absence of CNTFRα and the kinetics of the response to sCNTFRα indicate that the other components of the receptor complex contribute little, but measurably, to the specific potency of CNTF.

The human receptor for ciliary neurotrophic factor (CNTF) is a multicomponent system consisting of an extracellular ligand-binding protein (huCNTFRα) and two signal-transducing transmembrane proteins gp130 and LIFRβ. One or both of the latter are shared components of the receptors for LIF, IL-6, oncostatin M, and probably other cytokines (Davis *et al.*, 1991, 1993a, 1993b; Gearing *et al.*, 1992; Taga *et al.*, 1992; Ip *et al.*, 1992; Stahl *et al.*, 1993). huCNTFRα lacks intracellular and transmembrane domains and is anchored to the cell surface through a glycosyl phosphatidylinositol (GPI) tether (Davis *et al.*, 1991). However, recombinant "soluble" (sCNTFRα) purified by the method described here was shown to confer CNTF-dependent survival to normally nonresponsive, non-neuronal cell lines (Davis *et al.*, 1993a). In particular, addition of increasing amounts of sCNTFRα to human erythroleukemia TF-1 cells progressively lowered the amount of rat CNTF necessary to support their survival. This effect was apparently mediated by the reconstitution of complete receptor complexes on the surface of these cells which lack CNTFRα but have detectable levels of gp130 and LIFRβ mRNA (Davis *et al.*, 1993a). Increased levels of free receptor have also been detected after nerve injury, further suggesting that the activity of CNTFRα as a released factor is of physiological importance (Davis *et al.*, 1993a).

Because of this likely dual physiological role as a cell-surface receptor and a diffusible factor, it is important to study the ligand affinity and specificity of sCNTFRα and compare it with the cell-surface form. A characteristic property of cell-surface huCNTFRα is that it shows a significantly lower affinity for its homologous human CNTF ligand than for rat CNTF, as evidenced by an 8-fold weaker competitive ligand-binding inhibition and 4–5-fold lower specific biological activity (Masiakowski *et al.*, 1991; Panayotatos *et al.*, 1993). Interestingly, a single amino acid in human and rat CNTF is responsible for this ligand specificity (Panayotatos *et al.*, 1993). To best characterize the properties of sCNTFRα, we purified recombinant protein, studied its interaction with CNTF, and measured quantitatively its potency toward human and rat CNTF in a biological assay relevant to its potential humoral action. The results show that sCNTFRα as a diffusible factor maintains the relative ligand affinity and specificity of the cell-surface form and is the primary determinant of CNTF potency. This is the first report on the production and characterization of sCNTFRα.

MATERIALS AND METHODS

Expression Vectors for sCNTFRα. Plasmid pRPN249 was generated by replacing the DNA between the unique *Sal*1 and *Eag*1 restriction sites in pRPN40 (Masiakowski *et al.*, 1991) with a fragment amplified from plasmid pCMX–hCNTFR (Davis *et al.*, 1991), using suitable polymerase chain reaction (PCR) primers. The huCNTFRα gene in this fragment was designed to encode amino acids 22–334, i.e. nearly all of the proposed mature receptor sequence (Davis *et al.*, 1991). The exact position of the GPI link at the C-terminus is most likely located between positions 325 and 345, on the basis of recently proposed predictive rules (Kodukula *et al.*, 1993).

In earlier studies, two other expression vectors were engineered: pRPN151, encoding amino acids 22–349 but with cysteine at position 347 in accordance with the initial published sequence (Davis *et al.*, 1991), and pRPN245, encoding amino acids 22–349 with serine at position 347 according to the corrected sequence (Ip *et al.*, 1993). Even though the receptor molecules encoded in these plasmids have 6, 8, and 7 cysteine residues, respectively, their relative ligand affinity and specificity were found to be comparable (data not shown). The recombinant protein encoded in pRPN151 was used in early studies of the biological activity of the released form of CNTFRα (Davis *et al.*, 1991). The work described here was carried out with the protein encoded in pRPN249.

Refolding, Purification, and Characterization. Plasmid pRPN249, in which the huCNTFRα gene is under the transcriptional control of the *lac*UV5 promoter, was transfected into *Escherichia coli* K-12 strain RFJ26, a *lacI*^{q} prototroph (J. Fandl, Regeneron Pharmaceuticals). The receptor was quantitatively extracted from inclusion bodies in 8 M guanidinium chloride (GdnHCl) as described for other

* Corresponding author.

® Abstract published in *Advance ACS Abstracts,* April 15, 1994.

0006-2960/94/0433-5813$04.50/0

recombinant proteins (Masiakowski *et al.*, 1991) and dialyzed against 10 mM Tris-HCl, 5 mM EDTA, pH 8.5, 0.6 mg/mL of reduced glutathione, 0.12 mg/mL of oxidized glutathione for 24 h at 4 °C. At the end of dialysis, the material was filtered to remove traces of particulate matter and passed through a Sephacryl S-100 column (Pharmacia) in 100 mM Tris-HCl, 0.1 mM EDTA, 50 mM NaCl, pH 8.0 at a linear velocity of 0.4 cm/min. Fractions were analyzed by SDS–PAGE and pooled. The gel filtration pool was diluted twofold and loaded onto a Porous II/Q/P column (PerSeptive Biosystems) equilibrated in 25 mM Tris-HCl, pH 7.5. Bound receptor was eluted with eight column volumes of a linear 0–0.5 M NaCl gradient at a linear velocity of 5 cm/min. Fractions were analyzed by SDS–PAGE, pooled, and stored at –40 °C. The purified receptor prepared by this method was at least 95% pure with no individual contaminants over 1%, as determined by reducing SDS–PAGE and reverse-phase chromatography. The purified receptor migrated as a single band on nonreducing SDS–PAGE and native gels, evidence of conformational uniformity (data not shown and Figure 3).

Native Protein Gel Electrophoresis. Samples were brought to 10% glycerol, loaded onto 6% native gels in 100 mM Tris-HCl, 2 mM EDTA, 80 mM sodium borate, pH 8.3 and run at 150 V at room temperature for 100 min. Proteins were visualized by Coomassie staining.

Cell Survival Assays. EC_{50} values of TF-1 human erythroleukemia cells (Davis *et al.*, 1993a) were determined at half-maximal saturation from at least two independent dose–response curves.

Ligand Binding Assays. Proteins were mixed in 100 mM Tris-HCl, 0.1 mM EDTA, 50 mM NaCl, pH 8.0 and analyzed by either native gel electrophoresis or size-exclusion chromatography. Recombinant human CNTF, rat CNTF, and ch228 were purified as described (Panayotatos *et al.*, 1993). Their concentrations were determined using extinction coefficient values calculated according to Gill and Von Hippel (1989). For human CNTF the calculated (1.24) and the experimentally determined values (1.29; J. Labdon, Regeneron, personal communication) are in agreement. The calculated values for rat CNTF, ch228, and sCNTFRα are 1.19, 1.23, and 2.0, respectively. The relative amounts of these proteins observed on Coomassie stained gels are consistent with these values, and determinations of protein concentrations by Bradford assays are also in agreement within experimental error (data not shown).

RESULTS AND DISCUSSION

Protein Refolding. Soluble CNTFRα as encoded in pRPN249 is a mostly hydrophilic, weakly acidic protein (calculated MW, 35 123; p*I*, 6.5) that has six cysteine residues dispersed throughout its sequence. As is frequently the case with recombinant proteins expressed in *E. coli*, the receptor was found in inclusion bodies and was quantitatively extracted and refolded by dialysis against reduced and oxidized glutathione.

Recovery of correctly refolded protein was achieved by size-exclusion chromatography, whereby the receptor eluted in two peaks: one in the void volume and one in the volume expected for a 35-kDa protein (Figure 1A). This is more evident in Figure 1B, which shows the prominent sCNTFRα band admist the background of bacterial proteins whose sizes progressively decrease with the elution volume. Analysis by native gel electrophoresis (see below) revealed that sCNTFRα eluting in the void peak consisted of aggregated, inactive molecules, whereas correctly refolded, active receptor eluted

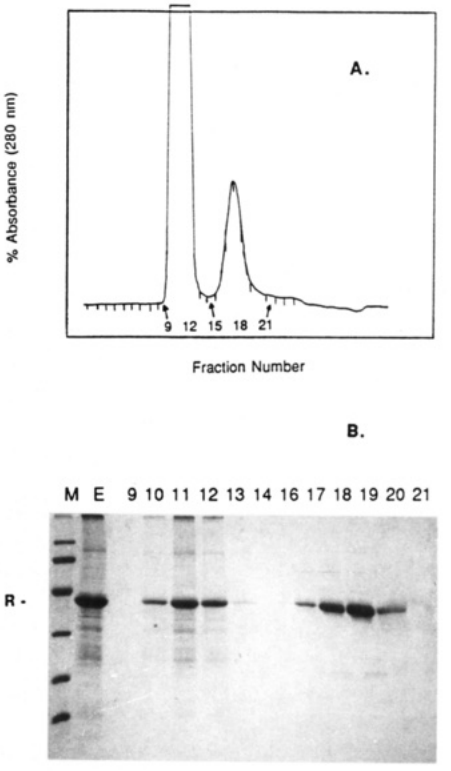

FIGURE 1: Separation of correctly refolded sCNTFRα: A, elution profile of a quantitative size-exclusion chromatography column monitored by absorbance at 280 nm; B, fractions collected in part A analyzed on reducing SDS–polyacrylamide gels. Total protein extract applied to the column (lane E) is also shown, along with size markers of 14, 21, 31, 45, 66, and 90 kDa (lane M). R- marks the sCNTFRα band at 35 kDa.

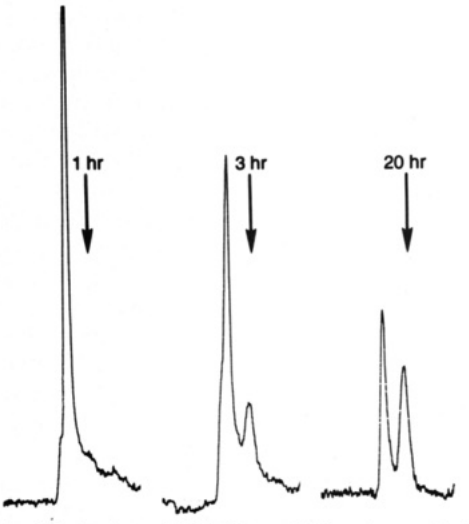

FIGURE 2: Monitoring sCNTFRα refolding by analytical size-exclusion chromatography. Aliquots of a refolding reaction mixture were removed at the indicated times and loaded on a Superdex 75 column. Elution profiles were monitored by absorbance at 280 nm.

in the 35-kDa peak. In fact, it was observed that during the course of the refolding reaction, the A_{280} ratio of the 35-kDa peak over the void peak progressively increased despite the fact that nucleic acids and other proteins contribute significantly to the absorbance of the void peak (Figure 2). Further analysis of receptor distribution in the two peaks by gel electrophoresis established that the progress and extent of refolding could be monitored simply from the intensity of the 35-kDa peak. Using this approach, the refolding conditions were optimized, eventually resulting in 70–90% recovery of

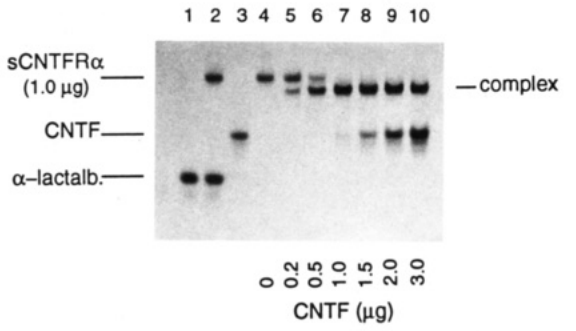

FIGURE 3: Receptor–ligand complex formation by native gel electrophoresis. A constant amount (1.0 μg) of sCNTFRα was mixed with the indicated amounts (in μg) of rat CNTF (lanes 4–10) or with 1 μg of α-lactalbumin as a control (lane 2): lane 1, 1 μg of α-lactalbumin; lane 3, 1.0 μg of rat CNTF.

correctly refolded recombinant sCNTFRα after refolding for 20 h (Figure 2).

Because the receptor contains many cysteine residues, one would normally denature and fully reduce the protein before refolding. Surprisingly, we observed that exposure of the receptor to reducing agents in the fully denatured state in 8 M GdnHCl led to poor yields of refolded protein. In fact, even addition of the redox agents to the 8 M GdnHCl extract immediately prior to dialysis against redox buffer gave poor results. However, reducing agents are required for correct refolding, since dialysis without redox agents also led to poor recoveries of refolded receptor. Optimal results were obtained by either the dialysis method described above or by incubation in redox agents in moderate concentrations of GdnHCl (data not shown). These results suggest that a disulfide bond(s) exists in receptor extracted in 8 M GdnHCl which is critical for refolding. Exposure to reducing agents at this stage disrupts this bond(s) and prevents refolding whereas, once the protein has been at least partially refolded, this bond(s) is protected and completion of disulfide bond formation requires redox agents. Even though the disulfide bond pattern of sCNTFRα has not yet been established, titration with Ellman's reagent did not detect the presence of free sulfhydryl groups, indicating that all six cysteines are disulfide bonded (data not shown).

Ligand-Binding Activity by Gel-Shift Assays. Evidence that the purified receptor represents active protein was obtained by analysis on nonreducing, nondenaturing (native) protein gels. Mobility on native gels is determined by the net charge (p*I*), molecular size, and shape of the protein. Accordingly, the fact that purified receptor migrates on native gels as one major band (Figure 3, lane 4) strongly indicated that the protein was refolded uniformly. Additional evidence of structural uniformity was obtained by reverse-phase HPLC and by nonreducing SDS–PAGE, whereby a single major protein species was detected (data not shown).

When sCNTFRα was mixed with increasing amounts of rat CNTF (pI, 5.7; MW, 22 721), the receptor band progressively disappeared and reappeared as a single band at a new position of the gel. Excess CNTF eventually appeared at the free-CNTF position (Figure 3, lanes 5–10). In control experiments, a band shift did not occur when rat CNTF was mixed with α-lactalbumin (Figure 3, lane 2) or with a number of other proteins not related to CNTF (data not shown). Thus, the protein-shift assay detects specific binding of sCNTFRα to its ligand, as expected from earlier applications of this method for detecting proteins that bind fluorescence-labeled actin (Safer *et al.*, 1989).

Stoichiometry of the huCNTFRα–CNTF Complex. The protein-shift assay can also be used to determine the approximate stoichiometry of a receptor–ligand complex. Figure 3 shows that both the transition of free receptor to the complex and the appearance of excess ligand occur over a narrow concentration range. Because of the mass difference, 1.0 μg of sCNTFRα should form a 1:1 complex with 0.65 μg of rat CNTF. No free ligand was seen when 1.0 μg of sCNTFRα was mixed with 0.2 or 0.5 μg of rat CNTF (lanes 5 and 6) whereas, upon 1.0 μg of sCNTFRα was mixed with 1.0, 1.5, 2.0, or 3.0 μg of rat CNTF, the amount of excess ligand expected from a 1:1 complex (0.35, 0.85, 1.35, and 2.35 μg, respectively) was seen (lanes 7–10). Therefore, these results indicate that, under these experimental conditions, sCNTFRα associated with its ligand in a 1:1 complex.

To obtain independent evidence of the binding stoichiometry, we analyzed complex formation by size exclusion chromatography. Figure 4 shows that the receptor–ligand complex elutes at the same position throughout the range of a 0.5–4.0 molar ratio, indicating that the size of the complex and, therefore, its stoichiometry remain constant. Moreover, the area of the complex absorption peak did not change in the 1.0–4.0 molar ratio, further indicating that excess CNTF did not alter the composition of the complex. Also, the observed size of the complex (69 kDa) and the amount of CNTF remaining unbound at each molar ratio are consistent with a 1:1 stoichiometry.

Theoretical calculations (Bazan, 1991) and mutational analysis (Panayotatos *et al.*, 1993) suggest that CNTF shares the structural organization of several cytokines, including growth hormone. This raised the possibility that CNTF, like growth hormone (Cunningham *et al.*, 1989; DeVos *et al.*, 1992), may also bind two receptor molecules through two distinct sites at low ligand–receptor ratios. The evidence presented above shows that this is not the case; both the relative amounts and the size of the ligand–receptor complex are consistent with one molecule of CNTF forming a complex with one molecule of receptor, regardless of the initial ratio at which the complex is formed. This conclusion, however, does not exclude the possibility that CNTF may also possess a second receptor binding site through which it forms a trimeric complex on the cell surface by binding a second but different receptor component, e.g. gp130 or LIFRβ. Such a heterotrimeric complex would be analogous to the 1:2 complex of growth hormone with its receptor and would be consistent with experimental evidence of the formation of such a complex on the cell surface (Davis *et al.*, 1993b).

sCNTFRα Potentiates Differentially the Response of TF-1 Cells to CNTF Variants. The activity of CNTFRα as a soluble factor was first demonstrated on TF-1 cells, where addition of sCNTFRα lowered the amount of rat CNTF necessary to support cell survival (Taga *et al.*, 1992). To further characterize the biological activity of sCNTFRα, we measured quantitatively the potentiation of rat CNTF-mediated survival of TF-1 cells and compared this value with that of human CNTF and a human CNTF point mutant (ch228) which displays the properties of rat CNTF (Panayotatos *et al.*, 1993). Figure 5 shows that added sCNTFRα strongly potentiates the effect on TF-1 cell survival of all three ligands. However, the shift in potency (EC_{50}) of human CNTF (Figure 5B) is much less pronounced than that of rat CNTF (Figure 5A) and ch228 (Figure 5C). Thus, sCNTFRα potentiates differentially the response of TF-1 cells to different CNTF variants.

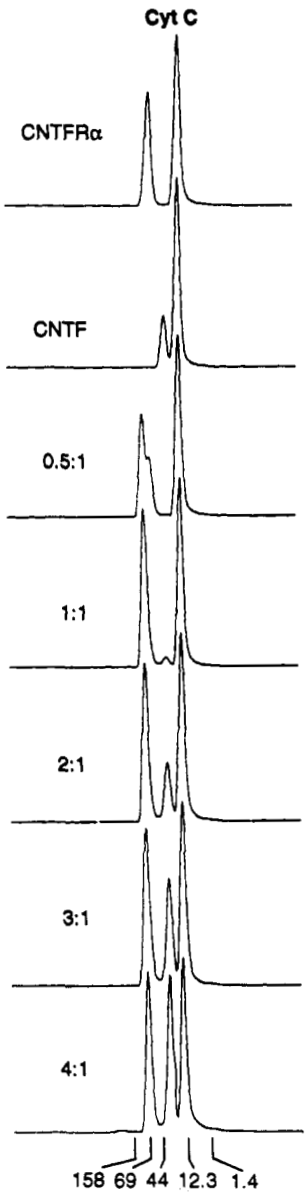

FIGURE 4: Stoichiometry of the sCNTFRα–ligand complex by size-exclusion chromatography. A constant amount of sCNTFRα (10 μM) was mixed with the indicated molar ratio of rat CNTF, and after 30 min at room temperature the samples were analyzed on a Superdex 200 column equilibrated and eluted with the same buffer at 76.4 cm/h linear velocity. Cytochrome *c* was added to each sample as an internal mobility reference. The positions at which the calibration markers of the indicated size (in kDa) elute from the column are marked at the bottom.

A more quantitative comparison of this differential potentiation of CNTF variants by sCNTFRα is shown in Figure 6. Plots of the EC_{50} values calculated for each of the CNTF response curves in Figure 5 vs. the concentration of sCNTFRα show an inversely proportional relationship (Figure 6A). Indeed, plots of $1/EC_{50}$ vs. [sCNTFRα] produced straight lines (Figure 6B) whose slopes provided a more quantitative measure of the interaction of sCNTFRα with each CNTF variant.

Because of the complexity of the system, it is difficult to draw conclusions from this kinetic behavior with regards to mechanism, but the linearity of the response is consistent with the notion that sCNTFRα and CNTF associate and interact with the cell surface components in a 1:1 complex. Regardless of mechanism, however, the value of each slope in Figure 6B reflects the effect of sCNTFRα on the potency (EC_{50}) of each

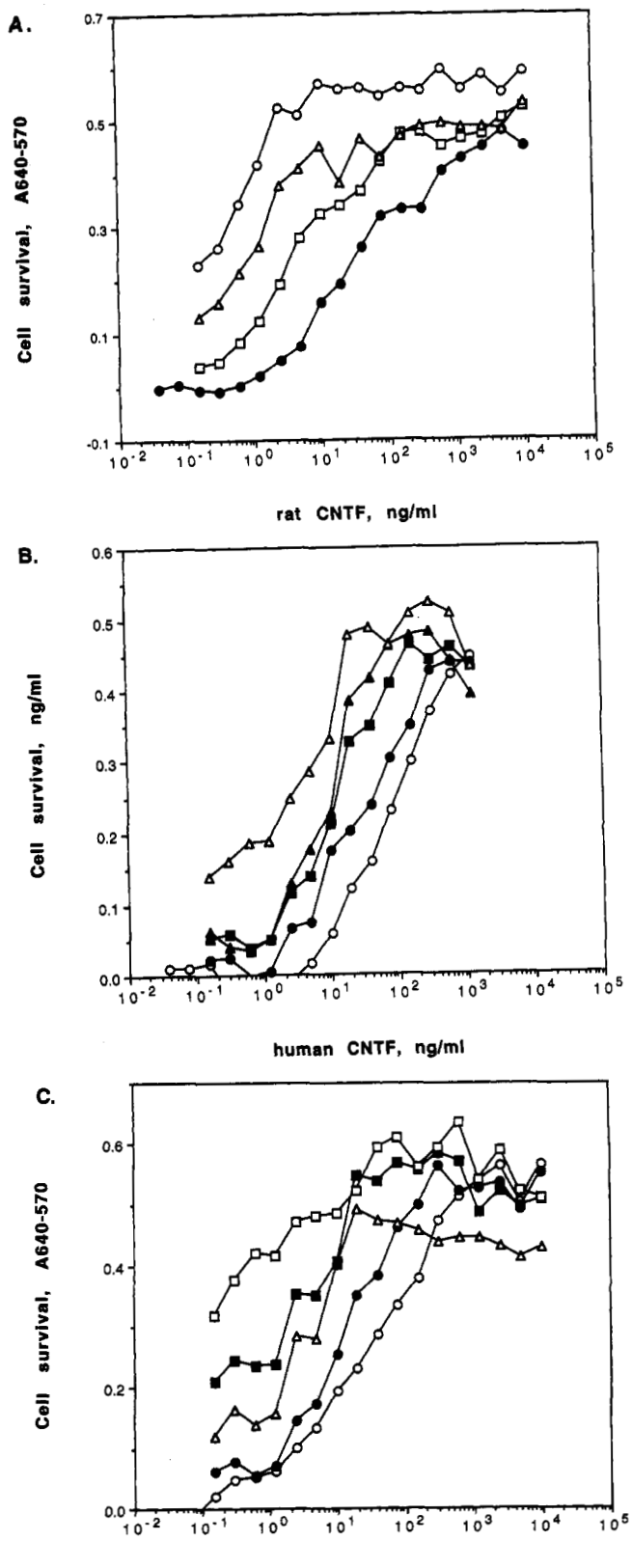

FIGURE 5: Potentiation by sCNTFRα of the effect of CNTF variants on TF-1 cell survival. Cells were grown in the presence of increasing concentrations of a CNTF ligand plus the indicated fixed concentrations sCNTFRα: A, rat CNTF, in the presence of 0 (filled circles), 10 (open squares), 50 (open triangles), and 250 (open circles) ng/mL of sCNTFRα, respectively; B, human CNTF in the presence of 0 (open circles), 20 (filled circles), 80 (filled squares), 200 (filled triangles), and 500 (open triangles) ng/mL of sCNTFRα, respectively; C, ch228 in the presence of 0 (open circles), 20 (filled circles), 80 (open triangles), 200 (filled squares), and 500 (open squares) ng/mL of sCNTFRα, respectively.

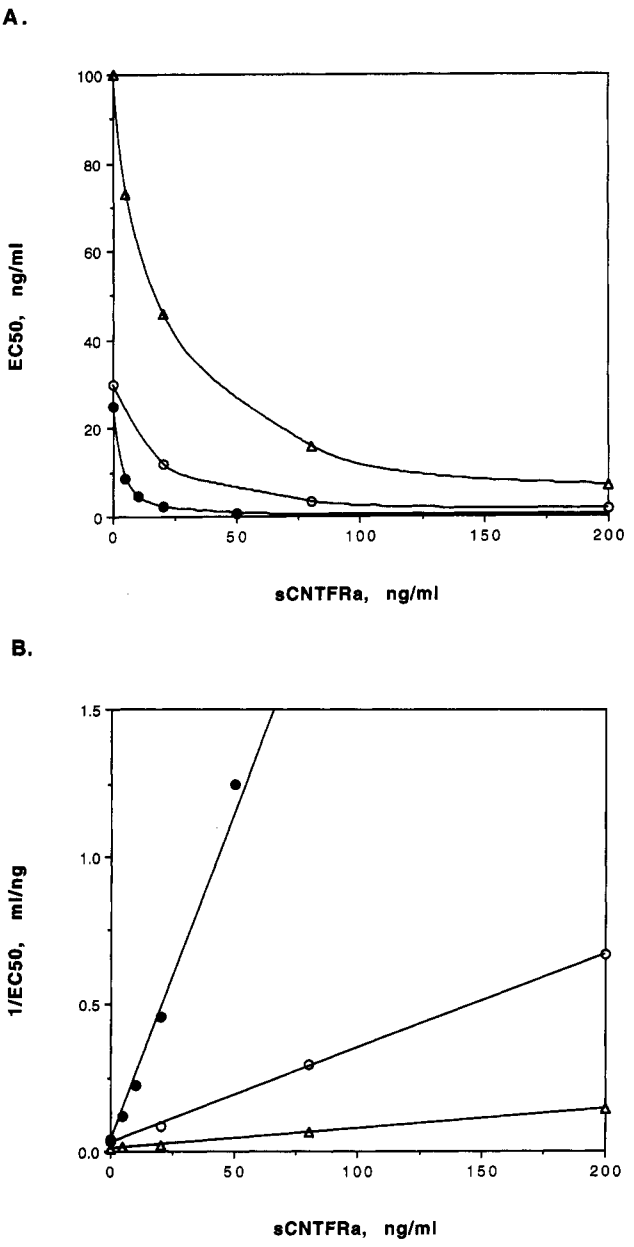

FIGURE 6: Determination of the relative ligand potency of sCNTFRα: A, EC_{50} values of each of the plots in Figure 5 plotted as a function of [sCNTFRα]; B, reciprocal plots of $1/EC_{50}$ vs. [sCNTFRα] of the same data. For both panels: rat CNTF, filled circles; ch228, open circles; human CNTF, open triangles.

ligand and the relative values of the slopes are a relative measure of the responsiveness of each CNTF variant to sCNTFRα. Naturally, for experiments carried out with a given CNTF species and different sCNTFRα variants, the slopes of the analogous curves would be a measure of the relative responsiveness of each sCNTFRα variant to CNTF. Therefore, this assay constitutes a quantitative method for measuring the specific activity of sCNTFRα and for evaluating its interaction with CNTF in a biologically relevant system.

Relevance to Cell Surface Receptor. A comparison of the values of the slopes obtained from Figure 6B shows that human sCNTFRα stimulates the response of the human TF-1 cells to rat CNTF and to ch228, many times more strongly than it stimulates the response to human CNTF (Table 1). Although unusual, the stronger stimulation of rat CNTF by sCNTFRα is, nevertheless, consistent with the relative biological potency of these ligands. Table 1 shows that rat

Table 1: Relative Affinity and Specific Activity of Human, Rat, and ch228 CNTF for sCNTFRα and Cell-Bound CNTFRα Measured by the Reciprocal Slopes of the Lines in Figure 6B; Competitive Binding toward MG87/CNTFRα Cells (Panayotatos *et al.*, 1993); E8 Chick Ciliary Neuron Survival (Panayotatos *et al.*, 1993); and TF-1 Cell Survival without Added Receptor

CNTF	slope × 10^4 (ng^2/mL^2)	MG87/Rα (IC_{50}; nM)	ciliary (EC_{50}; pM)	TF-1 (EC_{50}; nM)
human	14.9	25	9	8.0
rat	0.45	3	2	1.0
ch228	3.1	5	3	1.0

CNTF is 4–5 times more potent than human CNTF in its activity toward primary neurons, competes for binding to cell-surface-bound huCNTFRα 8–9 times more effectively than human CNTF (Masiakowski *et al.*, 1991; Panayotatos *et al.*, 1993), and is also 8 times more potent than human CNTF in supporting the survival of TF-1 cells in the absence of sCNTFRα. In addition, ch228 that displays rat CNTF-like properties is also 8 times more potent than human CNTF in the absence of sCNTFRα and is stimulated by sCNTFRα 5 times more strongly than human CNTF in the TF-1 assay (Table 1). Thus, the relative order by which sCNTFRα potentiates the effect of CNTF variants on TF-1 cell survival parallels both the relative affinity and the specific activity of these variants toward cell-surface receptor. Therefore, the purified recombinant receptor maintains the ligand preference of the cell-bound form and would be expected to maintain the same properties when acting as a diffusible factor.

Two other features of the response of TF-1 cells to CNTF and sCNTFRα are worth considering. First, in the absence of added sCNTFRα, TF-1 cells require for survival 8–9 times less rat CNTF or ch228 than human CNTF (EC_{50} = 25 ng/mL vs. 220 ng/mL, respectively). Second, even though addition of sCNTFRα shifts the EC_{50} of TF-1 cell response to CNTF by more than 1000 fold, the shift in the maximum number of surviving cells is marginal (Figure 5). This pattern indicates that although all cells eventually survive at high ligand concentrations, the activity of CNTF on TF-1 cells in the absence of exogenous receptor is transduced much less efficiently. This poor response could be due to pure signal transduction or low affinity binding of CNTF to a component other than huCNTFRα, most likely gp130 and/or LIFRβ. The latter explanation is consistent with the absence of detectable levels of huCNTFRα mRNA in TF-1 cells (Davis *et al.*, 1993a) and with cross-linking experiments indicating that CNTF is in close contact with gp130 and LIFRβ in the complete complex on the cell surface (Stahl *et al.*, 1993). Both of these facts suggest that other surface components in addition to huCNTFRα contribute to the affinity of CNTF for its receptor complex and eventually to the biological potency of CNTF.

REFERENCES

Bazan, J. F. (1991) *Neuron 2*, 197–208.

Cunningham, B. C., Ultsch, M., De Vos, A. M., Mulkerrin, M. G., Clauser, K. R., & Wells, J. A. (1989) *Science 254*, 821–825.

Davis, S., Aldrich, T. H., Valenzuela, D. M., Wong, V., Furth, M. E., Squinto, S. P., & Yancopoulos, G. D. (1991) *Science 253*, 59–63.

Davis, S., Aldrich, T. H., Ip, N., Stahl, N., Scherer, S., Farrugella, T., DiStefano, P., Curtis, R., Panayotatos, N., Gascan, H., Chevalier, S., & Yancopoulos, G. D. (1993a) *Science 259*, 1736–1739.

Davis, S., Aldrich, T. H., Stahl, N., Pan, L., Taga, T., Kishimoto, T., Ip, N. Y., & Yancopoulos, G. D. (1993b) *Science 260*, 1805–1808.

De Vos, A. M., Ultsch, M., & Kossiakoff, A. A. (1992) *Science 255*, 306–312.

Gearing, D. P., Comeau, M. R., Friend, D., Gimpel, S. D., Thut, C. J., McGourty, J., Brasher, K. K., King, J. A., Gillis, S., Mosley, B., Ziegler, S. F., & Cosman, D. (1992) *Science 255*, 1434–1437.

Gill, S. C., & von Hippel, P. H. (1989) *Anal. Biochem. 182*, 319–326.

Ip, N. Y., Nye, S. H., Boulton, T. G., Davis, S., Taga, T., Li, Y., Birren, S. J., Yasukawa, K., Kishimoto, T., Anderson, D. J., Stahl, N., & Yancopoulos, G. D. (1992) *Cell 69*, 1121–1125.

Ip, N. Y., McClain, J., Barrezueta, N., Aldrich, T. H., Pan, L., Li, Y., Wiegand, S., Friedman, B., Davis, S., & Yancopoulos, G. D. (1993) *Neuron 10*, 89–102.

Kodukula, K., Gerber, L. D., Amthauer, R., Brink, L., & Udenfriend, S. (1993) *J. Cell Biol. 120*, 657–664.

Masiakowski, P., Liu, H., Radziejewski, C., Lottspeich, F., Oberthuer, W., Wong, V., Lindsay, R. M., Furth, M. D., & Panayotatos, N. (1991) *J. Neurochem. 57*, 1003–1012.

Panayotatos, N., Radziejewska, E., Acheson, A., Pearsall, D., Thadani, A., & Wong, V. (1993) *J. Biol. Chem. 268*, 19000–19003.

Safer, D. (1989) *Anal. Biochem. 178*, 32–37.

Stahl, N., Davis, S., Wong, V., Taga, T., Kishimoto, T., Ip, N. Y., & Yancopoulos, G. D. (1993) *J. Biol. Chem. 268*, 7628–7631.

Taga, T., Narazaki, M., Yasukawa, A., Saito, T., Miki, D., Hamaguchi, M., Davis, S., Shoyab, M., Yancopoulos, G. D., & Kishimoto, T. (1992) *Proc. Natl. Acad. Sci. U.S.A. 89*, 10998–11001.