Exchange of a Single Amino Acid Interconverts the Specific Activity and Gel Mobility of Human and Rat Ciliary Neurotrophic Factors*

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Human and rat ciliary neurotrophic factors (CNTF), which share 85% sequence identity, promote the survival of chicken embryo ciliary ganglia neurons in vitro, but display a 4-5-fold difference in specific activity. To explore the origin of this difference and gain insight into the structural organization of CNTF, we created chimeric proteins of these two species. Surprisingly, we found that the differences in two apparently unrelated properties, gel mobility and specific activity, resided in a single amino acid. Substituting arginine residue 63 of rat CNTF into the human sequence created a protein with the properties of rat CNTF. Conversely, substituting the human CNTF glutamine residue 63 into rat CNTF generated a protein with the properties of human CNTF. Binding experiments confirmed that the distinct specific activities of human and rat CNTF and their chimeras reside in structural differences among these ligands rather than species differences in their receptors. Alanine substitution (Q63A) had no effect on the properties of human CNTF, whereas the R63A substitution reduced both the gel mobility and the specific activity of rat CNTF. Finally, a Q95R substitution at a different position of human CNTF had no effect on its properties. These results demonstrate that Arg-63 is both specific and critical in determining the structural differences of human and rat CNTF.

Despite the availability of recombinant ciliary neurotrophic factors (CNTF) from several species, little is yet known about their structure or their mode of interaction with their receptors. The rabbit (1), rat (2), and human (3) genes have been cloned and found to encode proteins that share about 80% sequence identity. Less conserved is the sequence of a chicken CNTF (also known as GPA (4)) which shows considerably lower homology (about 50%) to the proteins of the other three species. In addition, the receptor for human CNTF (CNTF receptorα) was cloned and shown to be part of a multicomponent system which shares the cell-surface protein gp130 and LIF receptor proteins with other cytokines (5, 6).

Human and rat CNTF differ in both their biological activity, receptor binding, and physical properties. The EC₅₀ of recombinant human CNTF in supporting survival and neurite outgrowth of embryonic chicken ciliary ganglion neurons in culture is 4-5-fold lower than that of rat CNTF (3). In addition, human CNTF also shows lower affinity for its homologous human receptor than rat CNTF (Footnote 2 and evidence shown below). Furthermore, despite the fact that recombinant human and rat CNTF have nearly identical mass (Mₑ ≈ 22,780 and 22,700 minus the NH₂-terminal methionine, respectively), their mobilities on denaturing and reducing SDS gels indicate a size difference of approximately 4,500 or 20%. These properties indicate that human and rat CNTF assume different structures both in the native and in the denatured state.

To identify the structural elements responsible for the above differences and to gain insight into the structural organization of CNTF, we exchanged corresponding segments of the human and rat CNTF gene sequences and studied the properties of the resulting chimeric proteins. Here we report that the nature of a single amino acid confers all of these properties.

MATERIALS AND METHODS

Genetic Engineering—The expression vectors pRPN33 and pRPN110 carry the human and rat CNTF genes, respectively (5). Restriction sites common to the two CNTF genes and unique in their corresponding expression vectors were introduced in areas that encode the same protein sequence in order to engineer the chimeric proteins shown in Fig. 1A. For example, pRPN219 was constructed from these vectors by exchanging the restriction fragment Mhel-HindIII of the human gene with the rat CNTF fragment of the same region, obtained by polymerase chain reaction amplification, using the primers RAT-III-dniH: 5' ACGTTAACGG'TAGGAGTCCTC 3'; and RAT-NHE-I-Mt 5' TCTATCTGGC TACGCAAAGG GATTCGC- TTCA GACCTGACT GTCTCCT 3'. The other plasmids described in this work were obtained by similar means, and their identity was confirmed by restriction analysis and DNA sequencing.

Protein Purification—Purification from inclusion bodies was as described for CNTF (3), with the occasional inclusion of a gel filtration step, and purification from cell lysates was as described for other proteins (7). Proteins were isolated to at least 80% purity, as seen in Fig. 1B. The key molecules, rat CNTF, human CNTF, and ch228 were purified to at least 95% purity, and their exact size was confirmed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (data not shown). Human CNTF has been shown to be sensitive to proteolytic cleavage at sites within the last 17 COOH-terminal residues (8). We assume that the closely spaced double bands seen for ch189, ch212, and ch233 in Fig. 1B are created by such cleavage during purification because they are not present in cell lysates. We have established that deletion of up to 22 residues from the COOH terminus does not alter the specific activity of CNTF.

Biological Activity Assays—CNTF was assayed on dissociated cultures of E8 chicken ciliary ganglion neurons as described (3), except that surviving cells were stained with 3-(4,5-diethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide and quantitated using a 450-nm absorbance signal. EC₅₀ values were determined at half-maximal saturation from the linear portion of at least two independent dose-response curves.

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1 The abbreviations used are: CNTF, ciliary neurotrophic factors; CG, ciliary ganglion; SCG, superior cervical ganglia.

2 N. Panayotatos et al., manuscript in preparation.

3 N. Panayotatos, E. Radziejewska, A. Acheson, D. Pearsall, A. Thadani, and V. Wong. unpublished results.
**RESULTS AND DISCUSSION**

*Chimeric CNTF Assays*—Fig. 1A shows an alignment of the primary structures of chimeric proteins sharing rat and human CNTF sequences. Theoretical calculations suggest that CNTF has the same structural organization around four amphipathic helices as found for several cytokines (12). In engineering chimeras, the boundaries of these regions (Fig. 1A) were taken into account so as not to mix residues from the rat and human sequences within any of the proposed helices, as that might not be compatible with the stricter structural requirements of a helix and might generate molecules of greatly distorted conformation.

Initially, we used the unique AlwN1 site. We created ch186 in which the first 52 amino acids of rat CNTF were substituted with the corresponding sequence of human CNTF, and the complementary molecule (ch187) in which the first 52 amino acids of human CNTF have been replaced by the corresponding rat CNTF sequence. Subsequently, we generated the complementary pairs ch188/ch189, where the segments between the Bsal restriction site and the COOH terminus of the human and rat CNTF genes were exchanged. Then, we created ch172, ch218, ch219, ch222, and ch224 where we gradually narrowed the rat CNTF sequences exchanged into human CNTF down to the single amino acid (Arg-63) of the rat sequence (Fig. 1A).

**Gel Mobility Differences**—A comparison of the gel mobilities of human, rat, and chimeric CNTF shows that ch186, ch189, ch219, and ch224 display the mobility of rat CNTF (Fig. 1B). Since the rat CNTF sequence is progressively limited in these chimeras down to a single arginine residue at position 63 (ch224), this

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**Fig. 1.** A, alignment of human, rat, and chimeric (ch186-ch228) CNTF proteins. Dots indicate residues of the human sequence. Unique restriction enzyme sites used in engineering the chimeras are shown, along with the four proposed amphipathic helices (indicated by *dashed* lines). B, mobility of human (H), rat (R), and the indicated purified chimeric and mutant CNTF molecules (ch186-ch236) on denaturing and reducing SDS-15% polyacrylamide gels. Lane M, markers of the indicated molecular weight.
result indicates that Arg-63 can singly confer higher gel mobility. Consistent with this result is the behavior of ch187, ch188, ch192, and ch222, which have a glutamine residue at position 63 and do not exhibit increased mobility, despite the presence of various other sequences of rat CNTF (Fig. 1B). Therefore, the single amino acid substitution (Q63R) is sufficient to confer to human CNTF the higher gel mobility of rat CNTF.

To confirm this result, we created ch235 (Table I). Despite the fact that this chimera consists of the entire rat CNTF sequence except for the human CNTF glutamine residue at position 63, it exhibits the mobility of human CNTF. Thus, the residue at position 63 can determine the mobility of CNTF, regardless of the species origin (rat or human) of the rest of the sequence.

To test whether the arginine or the glutamine residues were unique in their effects, point mutants of human (ch233) and rat CNTF (ch236) were constructed in which the residue at position 63 was substituted by alanine. This substitution did not affect the mobility of human CNTF but reduced the mobility of rat CNTF (Fig. 1B). Therefore, Arg-63 is rather specific in its effect.

Sequence Context of Arg-63 Is Responsible for Altered Gel Mobility—Is the strong effect on mobility (approximately 20% shift in apparent size) the consequence of any Q → R substitution or is it specific for amino acid 63? The fortuitous presence of a second Q → R variance at position 95 of the aligned CNTF sequences helped us answer this question. In contrast to Q63R, the Q95 → R substitution did not alter the mobility of ch192, which carries the Q95R (but not the Q63R) substitution and still exhibits the properties of human CNTF (Fig. 1B). Therefore, the effect on gel mobility of the Q63R substitution is specific and, as such, is not simply due to the structure, positive charge, SDS binding, or other properties of the arginine side chain. If this were the case, the same effect on mobility would have been seen with the chimera carrying the Q95R substitution, especially on denaturing SDS gels where all residues have free access to the solvent. The specific effect of the Q63R substitution on mobility must therefore originate in the primary structure context either around that residue or in other residues with which Arg-63 interacts. This sequence context most likely causes altered gel mobility by altering the amount of bound SDS, as proposed for other proteins (13).

Biological Activity and Receptor Binding Parallel Gel Mobility—In addition to gel mobility, the specific activity (EC₅₀) of each chimera was determined. In every case, the EC₅₀ value was at least comparable to human CNTF. However, Arg-63 chimeras had the lower EC₅₀ values of rat CNTF (Table II). For example, ch219 and ch228, which differ from the human CNTF sequence by only 4 and 1 amino acid, respectively, clearly displayed the biological activity of rat CNTF (Fig. 2). These results indicate that the amino acid at position 63 determines not only the difference in gel mobility

| Protein | Amino acid | Gel mobility | Specific activity | Binding inhibition |
|---------|------------|--------------|------------------|--------------------|
| Human   | Q          | hu           | 5.2              | 25                 |
| Rat     | R          | rat          | 1.2              | 0.8                |
| ch186   | R          | rat          | 1.0              | 6.0                |
| ch187   | Q          | hu           | 4.2              | 5.6                |
| ch188   | Q          | hu           | 4.8              | 8.6                |
| ch189   | R          | rat          | 2.8              | 3.6                |
| ch192   | Q          | hu           | 4.5              | 139                |
| ch218   | Q          | hu           | 4.2              | 15                 |
| ch219   | Q          | hu           | 4.2              | 15                 |
| ch222   | Q          | hu           | 4.2              | 15                 |
| ch223   | Q          | hu           | 4.2              | 15                 |
| ch228   | Q          | hu           | 4.2              | 15                 |
| ch229   | Q          | hu           | 4.2              | 15                 |

* Amino acid at position 63 of each protein.
* On SDS-PAGE, relative to human (hu) or rat CNTF.
* On E8 ciliary neurons (EC₅₀ pm).
* Toward MG87/CNTFRα cells (IC₅₀ nM). ND, not determined.

Fig. 2. Biological activity of human, rat, and two CNTF chimeras. A, ch219; B, ch228. Dose-response of dissociated E8 chick ciliary neurons surviving at the indicated protein concentration. Each experimental point represents the mean ± S.D. of three independent experiments.
but also the difference in the specific activity between rat and human CNTF.

Because chicken CNTF carries an arginine at position 63, the higher specific activity of rat relative to human CNTF on chicken neurons might merely reflect a stronger interaction of R63 proteins with the chicken receptor. To examine this possibility, we carried out receptor binding competition experiments with two types of cells: cultured neonatal rat superior cervical ganglion (SCG) neurons which normally respond to CNTF (14) and rat MG87 fibroblasts which only bind CNTF when they are transformed by a plasmid expressing the binding component (CNTFRA) of the human CNTF receptor (5, 6). In every case examined, Arg-63 chimeras showed stronger competition toward 125I-rat CNTF than Gln-63 chimeras. This pattern was observed with both types of cells, as can be seen from the competition curve for ch219 on SCG neurons (Fig. 3) and from the IC<sub>50</sub> values for ch228 on MG87/CNTFRA cells (Table I). These results indicate that the higher specific activity and binding potency of rat CNTF and Arg-63 chimeras are independent of the species origin of the receptor and must, therefore, reflect structural differences in the ligands rather than the receptors. This conclusion is consistent with evidence obtained with purified recombinant human CNTFRAs and human cell lines.

The relative specific activity and receptor binding of these chimeras parallel their gel mobility. To further test this relationship, we compared the properties of rat and human CNTF carrying arginine, glutamine, or alanine at position 63 (Table I). As observed with gel mobility, alanine substitution in human CNTF (ch233) has no effect on its activity. In contrast, glutamine (ch235) or alanine (ch236) substitution in rat CNTF lowers its gel mobility, specific activity, and binding potency to that of human CNTF. In fact, within experimental error (±40%), human CNTF, ch233, ch235, and ch236 have the same gel mobility, EC<sub>50</sub>, and IC<sub>50</sub> values, which are, as a group, distinct from those of rat CNTF and ch228. Therefore, the specific effect of the Arg-63 substitution on the specific activity and receptor competition of human CNTF is closely paralleled by its effect on gel mobility. This close relationship indicates that a structural perturbation involving Arg-63 underlies all three properties.

Mechanism—It may be unusual that an exchange of a single amino acid can interchange the specific activity of two proteins, but the fact that the same amino acid can alter their apparent size by 20% on denaturing SDS gels is intriguing. In the one case where a relationship between activity and gel mobility was observed, it was proposed that a conformation involving the relevant residues was stable to denaturation and was responsible for both phenomena (15, 16). This simple interpretation may also apply to the R63Q mutation. However, the alternative more complex possibility that the sequence context selectively alters SDS binding and gel mobility independently of the effect on receptor binding cannot be ruled out. The effect of the Q63R substitution on receptor binding indicates that Arg-63 optimizes receptor contact. Also, Arg-63 is located in the region between helix A and B that has been postulated to make direct contact with the receptor (12). These facts together suggest that either Arg-63 or other residues whose conformation is altered by Arg-63 make direct contact with the receptor. The nature and exact residues involved in this structure and their contributions to receptor binding, biological activity, and gel mobility are being explored.

Apart from its implications as to the possible structure of CNTF, the direct link between specific activity and gel mobility may be of practical advantage in the design of more potent therapeutic proteins. With the increasing availability of precisely engineered mutant proteins, a closer look at this phenomenon may be warranted. In this respect, the stronger binding of ch228 to the human receptor may translate to more potent pharmacological activity in vivo.

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**FIG. 3. Competitive ligand binding of human, rat, and ch219 toward SCG neurons. Vertical bars indicate standard deviation from the mean of three determinations. Non-specific binding background (250 cpm/well) has been subtracted from each point.**