Isolation and Characterization of Ciliary Neurotrophic Factor from Rabbit Sciatic Nerves*

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Ciliary neurotrophic factor (CNTF) has been purified 35,000-fold to homogeneity from rabbit sciatic nerves using its ability to promote the survival of chick embryo ciliary ganglion neurons as the bioassay. The purification involved a combination of acid treatment, ammonium sulfate fractionation, hydrophobic interaction chromatography, chromatofocusing, preparative sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and reversed-phase high performance liquid chromatography. Overlapping peptide sequences were obtained which accounted for 49% of the primary structure of the molecule. This information was used to prepare synthetic peptides in order to elicit antibodies. Purified CNTF exhibited two major and several minor bands between 21 and 22 kDa on silver-stained sodium dodecyl sulfate-polyacrylamide gel electrophoresis gels. All of the molecular forms were immunostained in Western blots by antiserum to synthetic peptides. The peptide sequences also provided a basis for cloning and expression of the rabbit CNTF gene (Lin, L-F. H., Mismer, D., Lile, J. D., Armes, L. G., Butler, E. T., III, Vannic, J. L., and Collins, F. (1989) Science 246, 1023-1025) confirming that the protein purified as reported here is CNTF.

The survival and phenotypic characteristics of developing neurons have been shown to depend on information derived from the innervated target. For sympathetic and neural crest-derived sensory neurons there is convincing evidence in cell culture and in animals to show that the trophic effect of the target is mediated by the well characterized protein, nerve growth factor (NGF) (see Ref. 1 for review). Clearly, models of neuronal development based on the existence of trophic factors to address cholinergic parasympathetic neurons, CNTF also promotes the survival in culture of the two classic NGF targets, sympathetic and sensory neurons (4). More recently CNTF has been shown to promote the survival and cholinergic differentiation of embryonic retinal neurons (5), to halt proliferation of sympathetic neuroblasts and drive their cholinergic differentiation (6, 7), and to regulate developmental specification of the type 2 astrocyte cell lineage in optic nerve (8-10). These findings suggest that CNTF is potentially important as a factor involved in the maintenance and differentiation of embryonic neurons and glia. CNTF may also play a role in the adult nervous system, since the highest concentration of CNTF activity in adult rodents has been detected in peripheral nerves (11, 12).

The molecular characterization of CNTF is a necessary first step in determining unequivocally its role in the embryonic and adult nervous systems. The survival-promoting activity of CNTF was first described in 1978 (13), and several years have passed since its ability to retain biological activity after reducing SDS-PAGE (12, 14) was used as the basis for its partial purification from avian eye (4) and rat sciatic nerves (15). However, the primary structure of CNTF has not been forthcoming. This report presents methods for the purification of CNTF from rabbit sciatic nerve tissue, the partial amino acid sequence of purified CNTF, and the production of specific antibodies to synthetic peptides based on this amino acid sequence. Oligonucleotide probes were derived from the amino acid sequence of CNTF and used to clone the CNTF gene; expression of the cloned gene in COS cells resulted in the synthesis of a protein with the biological and immunological characteristics of rabbit CNTF (16).

Experimental Procedures and Results

Criteria of CNTF Purity—Two bands were visible in silver-stained SDS-polyacrylamide gels of each of the bioactive

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* Portions of this paper (including "Experimental Procedures," part of "Results," Table I, and Figs. 1-9, 11, and 12) are presented in miniprint at the end of this paper. Miniprint is easily read with the aid of a standard magnifying glass. Full size photocopies are included in the microfilm edition of the Journal that is available from Wavely Press.
fragments from the final purification step of RP-HPLC (Fig. 5A, inset). Both of these bands were stained on immunoblots using antiserum raised to CNTF synthetic peptide A (Fig. 10, panel C). The specificity of immunostaining with antiserum to peptide A was demonstrated by the following observations. Preimmune serum failed to detect either band of purified CNTF (Fig. 10, panel B). Immunostaining of CNTF was abolished by preincubating the antiserum with excess peptide A (Fig. 10, panel D) but not with an equal concentration of a control (non-CNTF) peptide (Fig. 10, panel E). Furthermore, both bands reacted with antibodies purified on a peptide A affinity column (Fig. 10, panel F). These results indicate that both bands in RP-HPLC-purified CNTF are related in primary structure. Similar results were obtained using antiserum raised to synthetic CNTF peptides B and C (not shown).

Extracts of the preparative SDS gel strips from both sides of the peak CNTF bioactivity (Fig. 4, strips c and e) were subjected to RP-HPLC (Fig. 5, C and D), and the area of Asb traces containing the CNTF bioactivity was integrated and compared with that obtained from the gel slice containing the peak of CNTF activity (Fig. 4, strip d and Fig. 5A). There was a linear relationship between the CNTF bioactivity loaded onto the RP-HPLC column and the amount of protein emerging from the column in the position of CNTF (Fig. 11). This indicates that the specific activity is relatively constant across the peak of CNTF obtained from preparative SDS-PAGE. However, reducing SDS-PAGE analysis of the CNTF purified by RP-HPLC from different regions of the preparative SDS-PAGE peak demonstrated heterogeneity in molecular size. RP-HPLC-purified CNTF (Fig. 5C) from the higher molecular weight side of the preparative gel (Fig. 4, strip c) showed one major band that ran at approximately the same position as the larger of the doublet (Fig. 12A, lanes 1 and 2). RP-HPLC-purified CNTF (Fig. 5D) from the lower molecular weight side of the preparative gel (Fig. 4, strip e) showed several bands that migrated within 1000 daltons below the doublet (Fig. 12A, lane 3).

It was estimated that the single band, the doublet, and the multiple lower molecular weight bands (Fig. 12) constituted about 28, 63, and 9%, respectively, of the total recoverable CNTF protein. All such bands were recognized in Western blots using affinity-purified antibodies to synthetic peptide B (Fig. 12B) suggesting that the different molecular weight forms of CNTF are related at the primary structure level. Also, the relative constancy of specific bioactivity across the preparative SDS-PAGE peak of CNTF indicates that the multiple molecular weight bands of CNTF are all biologically active in addition to being related immunologically.

**Neuronal Specificity of Purified CNTF**—The partial neuronal specificity of purified CNTF was compared with that previously reported for less pure preparations (4, 15). Only CNTF purified through preparative SDS-PAGE (Table I, step 7) was available in sufficient quantities to perform these assays. Such CNTF supported the survival of E11 chick embryo sympathetic ganglion neurons with an apparent specific activity approximately 3.5-fold greater than observed for E8 parasympathetic ciliary ganglion neurons whereas the same material had little or no effect on the survival of E8 chick embryo dorsal root ganglion neurons (Table II). Step 7-purified CNTF also increased neuronal survival in cultures of E10–12 dorsal root ganglion neurons although with a specific activity significantly lower than for ciliary ganglion neurons and not possible to determine accurately with the limited amounts of CNTF available. These relative specific activities of CNTF on different neuronal populations are essentially the same as those reported for preparations of CNTF purified 400–800-fold from chick eye and sciatic nerve tissues, respectively (4, 15). Our preparative SDS-PAGE-purified CNTF showed little contaminating protein when chromatographed by RP-HPLC, suggesting that the previously reported survival-promoting effects of preparations containing CNTF (4) on these different neuronal populations are due to the activity of the same protein.

**DISCUSSION**

We report here the purification of CNTF from rabbit sciatic nerves, partial amino acid sequence analysis, and the generation of antibodies to synthetic peptides based on the amino acid sequence. This work provided a basis for cloning the rabbit CNTF gene, which has been reported elsewhere (16). The peptide sequences reported here are identical to the amino acid sequence predicted from a cDNA clone. In addition, antibodies to CNTF have been produced by expression of the gene transiently in COS cells (16) confirming that the protein purified as reported here is CNTF.

A 400–800-fold purification of CNTF was previously reported from embryonic chick eye (4) and adult rat sciatic nerves (15) via homogenization, DEAE-ion exchange chromatography, sucrose density gradient ultracentrifugation, and preparative SDS-PAGE. Poor recovery and/or diffuse migration of the bioactivity prevented us from using these published procedures to isolate CNTF from rabbit sciatic nerve extracts which necessitated the development of a different scheme to purify CNTF. The eight-step purification procedure presented...
here resulted in a 35,000-fold purification (Table I). There are several indications that the CNTF preparation is pure. First, the amount of protein under the activity peak emerging from RP-HPLC correlated well with the amount of bioactivity originally loaded onto the column (Fig. 11). Second, there are extensive overlapping peptide sequences obtained from digesting several CNTF preparations with various proteases, suggesting that there is one major protein in the preparation. Third, the addition of two purification steps (hydrophobic interaction chromatography and fast protein liquid chromatography-hydrophobic interaction chromatography) failed to improve the final specific activity, and finally antibodies raised against three different synthetic CNTF peptides reacted specifically with all the bands visible in silver-stained gels of purified CNTF suggesting that the preparation is homogeneous with respect to the core primary structure.

In general agreement with previously published estimates (4, 12, 15), our results indicate that CNTF is an acidic protein with a molecular mass, determined from SDS-PAGE, of approximately 24 kDa. However, our results also indicate that purified CNTF is heterogeneous with respect to both charge and apparent molecular weight. Two major and several minor silver-stained bands were visible between 24 and 22 kDa in different fractions of purified CNTF (Fig. 12A). Each of these bands was recognized by affinity-purified antibodies to a number of synthetic CNTF peptides (Fig. 12B), and each of these fractions exhibited approximately the same specific activity in the CNTF biological assay (Fig. 11). This indicates that the multiple molecular weight forms observed are related to primary structure and biological activity and probably represent partial proteolytic fragments of CNTF. In addition, the results of chromatofocusing (Fig. 2) and RP-HPLC (Fig. 5) indicate heterogeneity in charge and hydrophobicity or conformation, respectively. It is unclear whether some or all of these observed heterogeneities exist in vivo or arise during the process of extraction and purification.

Also noteworthy among the attributes of CNTF is the relative resistance of its biological activity to harsh treatments including exposure to pH 3.6, exposure to the acidic organic solvents used in RP-HPLC, and heating in reducing SDS-PAGE sample buffer. This characteristic of CNTF was crucial to its complete purification.

CNTF does not appear to be extensively glycosylated. No sites suitable for N-linked glycosylation exist in the protein (16). In early experiments, we found that over 90% of the CNTF activity in the crude extract of rabbit sciatic nerves was not retained by passage of the crude extract through a concanavalin A or lentil lectin-Sepharose column, and no column with α-methylmannoside. Furthermore, the sharpness of the silver-stained and Western immunoreactive bands of the purified CNTF (Figs. 5A, inset, 10, and 12) also argues against any substantial glycosylation of the protein. Finally, CNTF migrates on SDS-PAGE in the position expected for its amino acid composition (16) within experimental error.

Our purified material possessed the biological specificity that was previously reported for partially purified CNTF (4, 15). Our preparations promoted the survival of E11 chick embryo dorsal root ganglion neurons in addition to the E8 parasym pathetic ganglion neurons in addition to the E8 parasym pathetic ganglion neurons routinely used in the bioassay (Table II). Also in accordance with previous reports, our purified CNTF was unable to promote the survival of E8 chick embryo dorsal root ganglion neurons. These results also indicate that CNTF is a potent neurotrophic factor that exerts its biological effects at concentrations significantly lower than for NGF or basic fibroblast growth factor (Table II). RP-HPLC-purified CNTF had an estimated specific activity of 2000 mU/mg, which indicates that half-maximal survival of ciliary ganglion neurons occurs at the relatively low concentration of approximately 2 pM. The estimated concentration of CNTF promoting half-maximal survival of E11 sympathetic ganglion neurons was even lower by a factor of approximately 3.5 (Table II). The specific activity measured for RP-HPLC-purified CNTF may even be underestimated somewhat due to partial inactivation of CNTF during exposure to the acidified organic solvents used in RP-HPLC.

The antibodies to synthetic CNTF peptides have proved useful in Western blot immunostaining of purified (Figs. 10 and 12) and recombinant (16) CNTF. However, none of the antibodies to synthetic peptides obtained so far has demonstrated an ability to immunoprecipitate or neutralize the native protein. It is possible that the epitopes recognized by these antibodies are only available after the denaturation associated with Western blot analysis. The availability of cDNA clones for CNTF will allow production of sufficient recombinant CNTF to generate monoclonal and polyclonal antibodies to the intact protein. If neutralizing antibodies can be generated in this way, they will be valuable tools for characterizing the functional role of CNTF in vivo.

Acknowledgments—We thank J. Forney, J. McDonald, and C. Squires for critical comments on the manuscript, and W. Heim for its preparation.

REFERENCES

METHODS

I. Preparation of Crystalline 8-10,000 wt% of ribose soybean (about 400 ng) were
precipitated with aqueous sodium acetate to collect crystals by centrifugation. The
supernatant containing the protein was centrifuged at 6000 g for 10 minutes and
washed with 500 mM sodium acetate pH 6.0. The crystals were mounted on a
carbon coated grid and viewed under transmission electron microscopy. The
particles were then analyzed by negative-stain electron microscopy.

II. Purification of Crystalline 8-10,000 wt% of ribose soybean (about 400 ng) were
precipitated with aqueous sodium acetate to collect crystals by centrifugation. The
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washed with 500 mM sodium acetate pH 6.0. The crystals were mounted on a
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V. Purification of Crystalline 8-10,000 wt% of ribose soybean (about 400 ng) were
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particles were then analyzed by negative-stain electron microscopy.
CNTF Purification

Fig. 1: HIC. The experimental procedures are described in "Methods". Fractions indicated by a bar were pooled.

Fig. 2: FPLC - Chromatofocusing. The experimental procedures are described in "Methods". The arrow indicates sample injection. The bar indicates the pool of active fractions.

Fig. 3: FPLC-HIC. The experimental procedures are described in "Methods". The arrow indicates sample injection. The bar indicates the pool of active fractions.

Fig. 4: Distribution of CNTF activity in gel electrophoresis from preparative SDS-PAGE. The region of the gel between 27-28 kDa was cut across the width into seven parallel strips (labeled a to g in order of decreasing molecular weight) using the banding patterns revealed by the SDS-stained microcinchomane paper (see "Methods"). Aliquots of the eluates from each gel strip were assayed for bioactivity.

Fig. 5: RP-HPLC of the gel eluate from preparative SDS-PAGE. The gel eluate from Fig. 4, strip d (Panel A), strip f (Panel C) or strip g (Panel D) was loaded onto a C4 column as described in "Methods" and eluted with the gradient indicated. Solvent A was 0.1% TFA in H2O and solvent B was 0.1% TFA in acetonitrile. Arrowheads indicate the pool of active fractions.

Fig. 6: SDS-PAGE gel of aliquots from indicated fractions. Arrowheads designate the 244Dx position.

Table I

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<th>Yield</th>
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<td>2000</td>
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<td>2. Acid Treatment</td>
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<td>1648</td>
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<td>3. Ammonium Sulfate</td>
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<td>22.3</td>
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n.d. = not determined
### Peptide Sequences

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<th>Sequence</th>
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<td>Glu-C-1,2,3</td>
<td>SALTPEREK</td>
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<tr>
<td>Asp-N-3</td>
<td>DQWURFPAEC</td>
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<tr>
<td>Asp-N-2</td>
<td>DGTYPVIGG</td>
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<tr>
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<td>DOLFKEL</td>
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<td>Asp-N-6,7</td>
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### PEPTIDE SEQUENCE

| LARKQSDLTAETSSYNEGLVNNHKHLDHSVGDQVPA |
| Lys-C-4 |
| Asp-N-2 |
| Arg-N-1 |
| Glu-C-1 |
| Asp-N-3 |
| Chymo-3 |
| Chymo-2 |
| Lys-C-1 |
| Asp-N-1 |

### Summary of the Proof of the Polypeptide Sequence of CNTF

The outlined sequences of specific peptides are indicated by a solid line. Fragments Lys-C, Lys-9, Lys-10, and Glu-C denote peptides sequenced by digestion with cyanogen bromide, endoproteasome Lys-C, Arg-N, and Glu-C, respectively. Peptides are numbered sequentially as they appeared in the N-HPLC (Fig. 9). All peptide sequences were determined by Edman degradation. Peptides A, B, and C as indicated by dotted lines were synthesized chemically for use as antigens.

### Reactivity in the Chan's or Preparative SDS-PAGE gel is proportional to the protein recovered from RP-HPLC.

The TU recovered from gel shifts A, B, and C in Fig. 4 was plotted against the protein recovered under the isoelectric point in Fig. 10. ANA, and BSA.

### Fig. 11. Reactivity in the Chan's or Preparative SDS-PAGE gel is proportional to the protein recovered from RP-HPLC.

The TU recovered from gel shifts A, B, and C in Fig. 4 was plotted against the protein recovered under the isoelectric point in Fig. 10. ANA, and BSA.

### Fig. 12. SDS-PAGE and Western blot analysis of purified CNTF.

Samples in panel A and B are: lane 1, 1/5 of fraction #48 of Fig. 7; lane 2, 1/50 of fraction #48 of Fig. 7; lane 3, 1/5 of fraction #48 of Fig. 7; lane 4, molecular weight standards, from top: 66, 45, 36, 29, 20, and 14.2 kDa. Western blot was with affinity-purified anti-peptide antibodies.
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