Purification, Cloning, and Expression of Ciliary Neurotrophic Factor (CNTF)

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Ciliary neurotrophic factor (CNTF) is one of a small number of proteins with neurotrophic activities distinct from nerve growth factor (NGF). CNTF has now been purified and cloned and the primary structure of CNTF from rabbit sciatic nerve has been determined. Biologically active CNTF has been transiently expressed from a rabbit complementary DNA clone. CNTF is a neural effector without significant sequence homologies to any previously reported protein.

THE CHEMICAL AND NEUROTROPHIC PROPERTIES OF CNTF distinguish it from the only well-characterized neurotrophic factor, NGF, and suggest that it has an important role in neural development. Substantially purified preparations of CNTF promote the survival in culture of neurons from embryonic parasympathetic, sympathetic, and sensory ganglia (*1*). Similar preparations promote the differentiation of sympathetic neuroblasts by inhibiting their proliferation and increasing the levels of neurotransmitter and transmitter synthetic enzymes (*2*). CNTF has also been implicated in regulating the differentiation of the type-2 astrocyte cell lineage during development of the optic nerve (*3*). These activities are associated with an acidic protein of about 24 kD that is found in highest concentrations in peripheral nerve tissue and which has biological activity that survives reducing SDS–polyacrylamide gel electrophoresis (*4*).

To characterize the protein responsible for these activities, we purified CNTF from adult rabbit sciatic nerves (*5*) with the bioassay originally used to define CNTF: its ability to promote the survival of chick embryo ciliary ganglion neurons (*6*). A final specific activity of 12 trophic units (TU) per nanogram was observed after an approximately 25,000-fold purification. The concentration of TU per milliliter was defined as the reciprocal of the dilution that gave 50 percent of maximal survival in the ciliary ganglion survival assay (*6*).

We could not obtain an amino-terminal amino acid sequence from the intact protein. Therefore, peptides generated by various proteolytic digestions were purified and sequenced (*7*). Stretches of primary structure derived from overlapping peptide sequences are underlined in Fig. 1.

We used the polymerase chain reaction (PCR) (*8*) to obtain just over half the sequence of the rabbit gene for CNTF. Degenerate oligonucleotides based on the sequences indicated as 1, 2, and 3 in Fig. 1 were synthesized in sense and antisense orientations. PCR was performed with rabbit genomic DNA or rabbit sciatic nerve complementary DNA (*9*) as template and oligonucleotides 1 (sense) and 3 (antisense) as primers. The products from each reaction were subcloned into a single-stranded phage vector and screened with labeled intervening oligonucleotide 2 (*8*). Several oligonucleotide 2 positive clones were sequenced, each giving the sequence of the rabbit gene between oligonucleotides 1 and 3 (Fig. 1).

The insert from one such clone was labeled and used to screen 200,000 independent clones of a rabbit sciatic nerve cDNA library (*9*), yielding a single positive clone. This clone was digested with the restriction enzyme Eco RI, resulting in three fragments of 2.0, 1.5, and 0.6 kb, which made up the entire insert. The 1.5-kb fragment was subcloned and sequenced in both orientations (*9*) and found to contain the entire coding sequence for rabbit CNTF (Fig. 1). The sequence obtained agrees exactly with amino acid sequences obtained from purified CNTF (Fig. 1) and with the DNA sequences obtained with PCR. The CNTF sequence deduced from the cDNA has a calculated molecular size of 22.7 kD and a calculated isoelectric

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M A F M E H S A L T P H R R E L C S R T I W L A R K I R S D      30
L T A L T E S [Y V K H Q G]1 L [N K N I N L]2 D S V D G V P M A S  60
T D Q W S E L T E A E R L Q E N L Q A Y R T F H I M L A R L      90
L E D Q Q V H F T P A E G D H F Q A I H T L L L Q V A A F A      120
Y Q I E E L M V L L E C N I P P K D A D G T P V I G G D G L      150
F E K [K L W G L K]3 V L Q E L S H W T V R S I H D L R V I S C  180
H Q T G I P A H G S H Y I A N D K E M STOP                      199
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Fig. 1. The inferred amino acid sequence for rabbit CNTF. The underlined amino acid sequences are identical to those obtained by sequencing purified CNTF. The sequence is considered complete because there is a stop codon three base pairs upstream of the presumed initiating methionine and another stop codon immediately after the carboxyl-terminal methionine. In the absence of amino-terminal amino acid sequence, the first methionine following the upstream stop codon was assigned as the site of initiation of translation. The amino acid sequences used to generate several degenerate oligonucleotides are boxed. The nucleic acid sequence has been deposited with GenBank: accession number M29828. Abbreviations for the amino acid residues are as follows: A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; W, Trp; and Y, Tyr.

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point (*p*I) of 5.78, both of which are in good agreement with direct measurement of these properties in the purified protein.

The 1.5-kb Eco RI restriction fragment containing the coding sequence for CNTF was used to probe an RNA (Northern) blot of polyadenylated RNA from rabbit sciatic nerve. It hybridized to a band of approximately 4.3 kb (Fig. 2). The 2.0- and 0.6-kb Eco RI restriction fragments from the cDNA clone recognized the same 4.3-kb band when used to probe this RNA blot, suggesting that the cDNA insert (4.1 kb) is approximately full length and that all three Eco RI fragments are part of the same cDNA. There was no hybridizing RNA detected when the 1.5-kb Eco RI fragment was used to probe polyadenylated liver RNA (Fig. 2), suggesting tissue specificity in the distribution of this message.

Biologically active recombinant CNTF was expressed by using rabbit sciatic nerve cDNA. The 1.5-kb Eco RI restriction fragment was subcloned into a transient expression vector and transfected into COS-7 cells (*10*). After 48 hours the cell extract, but not the culture medium, contained significant CNTF biological activity (Fig. 3). In contrast, control COS-7 cell cultures transfected with expression vector without an insert had no detectable biological activity in either the cell extract or culture medium.

Protein immunoblots of the biologically active extract of cells transfected with the rabbit cDNA showed a single band at about 24 kD, which reacted with affinity-purified antibodies to CNTF peptides (Fig. 4, lane 8). This band was not detectable in the biologically inactive extract of control cells transfected with vector alone (Fig. 4, lane 10) or in the biologically inactive culture media from cells transfected with cDNA or vector only. Thus, there was a strong correlation between the presence of CNTF biological activity and the appearance of a CNTF cross-reacting species. The specific activity of CNTF produced by COS-7 cells was roughly estimated from bioactivity and protein immunoblots to be 10 to 15 TU/ng, which is consistent with the average specific activity of purified CNTF (12 TU/ng).

Searches of protein and nucleic acid sequence banks (*11*) revealed no significant homologies between CNTF and any other reported sequences, including other proteins with neurotrophic activities such as acidic and basic fibroblast growth factor (*12*), neuroleukin (*13*), purpurin (*14*), and NGF (*15*). In addition, the primary structure of CNTF does not contain an amino-terminal signal sequence of the type that is typically cleaved in secreted proteins (*16*). Possibly as a consequence of this, CNTF was detected only in cell extracts, not culture medium, when transiently expressed in COS-7 cells (Fig. 3). In contrast, NGF has an amino-terminal signal sequence that is removed during processing and secretion of the mature protein (*15*).

In summary, we have purified CNTF from rabbit sciatic nerves and obtained partial amino acid sequence which was used, in

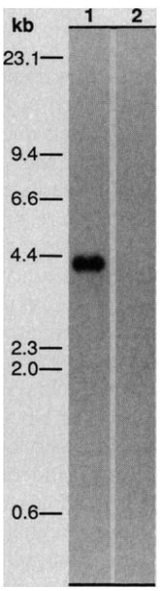

Fig. 2. RNA (Northern) blot analysis with rabbit sciatic nerve and liver RNA. Polyadenylated RNA (*9*) from rabbit sciatic nerves (lane 1, 5 μg) and rabbit liver (lane 2, 7.5 μg) were electrophoresed on a 1 percent agarose, 6.6 percent formaldehyde gel and transferred to Zeta-Probe blotting membrane (Bio-Rad) and cross-linked with ultraviolet irradiation. The 1.5-kb Eco RI restriction fragment (see text) was ^{32}P-labeled by random priming (*9*) to 1.5×10^8 dpm/μg and hybridized to the blot for 16 hours at 42°C in 5× saline sodium citrate (SSC) (1× SSC is 150 m*M* sodium chloride, 15 m*M* sodium citrate) containing 50 percent formamide, 7 percent SDS, yeast tRNA (0.15 mg/ml), and sheared salmon sperm DNA (0.05 mg/ml). The blot was washed in 0.2× SSC containing 0.5 percent SDS at 65°C and exposed to Xomat AR50 film (Kodak) for 6 hours at −80°C with a Cronex Quanta 3 intensifying screen. The size markers were Hind III restriction fragments of DNA from phage lambda.

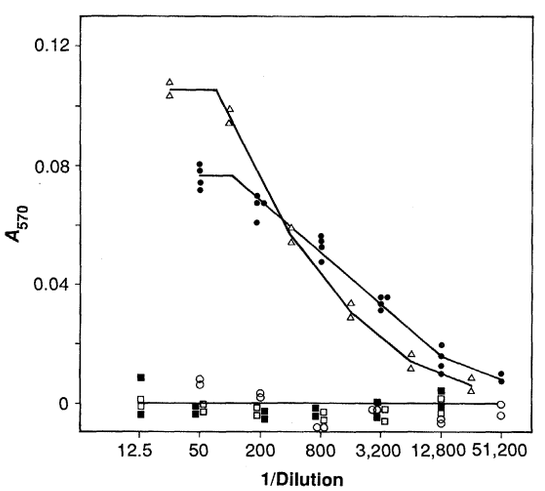

Fig. 3. Transient expression of CNTF. COS-7 cells were transfected with a transient expression vector containing rabbit CNTF cDNA (●, ○) or vector alone (■, □). After 48 hours, serum-free culture medium containing 0.4 μg of protein per milliliter (○, □) and cell extracts, obtained by brief sonication of cell pellets, containing 0.3 mg of protein per milliliter (●, ■) were bioassayed (*6*). Absorbance at 570 nm (increasing neuronal survival) in replicate wells is plotted as a function of the final dilution of samples. Rabbit sciatic nerve extract at 1.4 mg of protein per milliliter was assayed as a positive control (△).

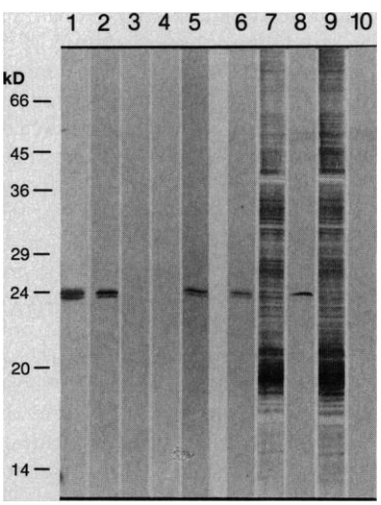

Fig. 4. (Lanes 1–6) The specificity of antibodies to CNTF. Purified CNTF (20 ng/lane) exhibited two major (and often several closely spaced minor) bands between 22 and 24 kD after silver-staining (lane 1). All such bands were immunostained in protein immunoblots (*17*) by antiserum to synthetic CNTF peptide A (*18*) (lane 2). Immunostaining was specific because serum from unimmunized animals did not recognize purified CNTF (lane 3). Also, immunostaining by antiserum to peptide A was prevented by 0.6 m*M* peptide A (lane 4) but not by unrelated peptide at 0.6 m*M* (lane 5). Finally, antibodies purified on a peptide A affinity column (*18*) still immunostained CNTF (lane 6). Identical results (not shown) were obtained with antiserum to CNTF peptides B and C (*18*). (Lanes 7–10) The expression of CNTF immunoreactive material in COS-7 cells. Lanes 7 and 8 contain 3 and 60 μg of protein, respectively, from extracts of COS-7 cells transfected with rabbit CNTF cDNA in expression vector; lane 7 is silver-stained, whereas lane 8 is immunostained with antibodies purified on a peptide A affinity column. Lanes 9 and 10 are identical to 7 and 8, respectively, except that each contains the extract of control cells transfected with expression vector alone. Only cells transfected with CNTF cDNA contained material recognized by antibodies to CNTF peptides. In side-by-side comparisons, this immunostained band migrated in the position of the largest of the multiple, immunologically related bands in purified CNTF.

conjunction with PCR, to clone an approximately full-length cDNA for this protein. The primary structure of CNTF has been determined from this cDNA, and it agrees exactly with the sequences obtained both from the purified protein and from PCR. Bioactive and immunoreactive CNTF has been transiently expressed with the cDNA, confirming its identity. Our work provides a basis for defining at the molecular level the actions and functions of CNTF in the nervous system.

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5. Sciatic nerves (Pel-Freez Biologicals) were extracted in 10 ml of water per gram of tissue containing 10 m*M* EDTA, 1 m*M* ϵ-aminocaproic acid, 1 m*M* benzamidine, and 0.1 m*M* phenylmethyl sulfonyl fluoride. The extract was centrifuged at 140,000*g* for 30 minutes at 4°C, and the supernatant was filtered through glass wool to remove floating lipid. Precipitates forming after adjustment of the *p*H first to 3.6 and then to 6.3 were removed by centrifugation (17,000*g* for 20 minutes). The supernatant was then precipitated with 30 to 60 percent saturated ammonium sulfate; the precipitate was then dissolved in 0.3*M* ammonium sulfate, 5 percent isopropanol, 10 m*M* sodium phosphate buffer, *p*H 6.7, and applied to a column of phenyl–Sepharose CL-4B (Pharmacia). CNTF was eluted in 10 m*M* sodium phosphate buffer, *p*H 6.7, containing 50 percent ethylene glycol. Active fractions were concentrated, dialyzed against 25 m*M* bis-tris-HCl buffer, *p*H 5.8, and applied to a Mono-P fast protein liquid chromatography (FPLC) chromatofocusing column (Pharmacia) eluted with Polybuffer 74 (1:10), *p*H 4.0. Active fractions received solid ammonium sulfate to 95 percent saturation. The precipitate was dissolved in 1.5*M* ammonium sulfate in 50 m*M* sodium phosphate buffer, *p*H 6.7, and placed on an Alkyl-Superose HR10/10 FPLC column (Pharmacia) equilibrated with this buffer. The column was eluted with a linear gradient of 1.5 *M* to 0*M* ammonium sulfate in 50 m*M* sodium phosphate buffer, *p*H 6.7, and fractions containing bioactivity were pooled, concentrated, and analyzed on a 15 percent polyacrylamide resolving slab gel overlaid with a 3.75 percent polyacrylamide stacking gel [U. K. Laemmli, *Nature* **227**, 680 (1970)]. Narrow strips were cut across the gel between 22 and 27 kD and extracted in 0.01 percent Tween-20 in 5 m*M* sodium phosphate buffer, *p*H 6.7, at 4°C overnight. Gel extracts were placed in 2 percent dithiothreitol and 0.3 percent trifluoroacetic acid (TFA) onto a C8 reversed-phase high-performance liquid chromatography (HPLC) column (Synchrom) and eluted with a gradient of 0 to 100 percent acetonitrile in 0.1 percent TFA. Fractions containing bioactivity were concentrated and stored at −80°C. Protein was measured by the method of M. Bradford [*Anal. Biochem.* **72**, 248 (1976)] or by absorbance at 215 nm. The final recovery of CNTF was about 1.5 percent of the starting bioactivity. The yield was approximately 2 μg of CNTF protein for every 200 g (wet weight) of nerves. A partial purification of CNTF was previously reported (*1*).
6. Survival of ciliary ganglion neurons was assayed as described [F. Collins and J. D. Lile, *Brain Res.* **502**, 99 (1989)]. Briefly, ciliary ganglia were removed from 8- to 9-day-old chicken embryos, dissociated, and plated in 96-well microtiter tissue culture dishes at 1500 to 2000 neurons per well. Serial dilutions of the samples to be assayed were added, and incubation was continued at 37°C for 18 to 20 hours. At this time 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (0.3 mg/ml) was added, and the dishes were incubated for 4 hours at 37°C. MTT is converted to a blue product only in metabolically active cells, resulting in an optical density reading (570 nm) that is proportional to the number of surviving neurons.
7. Fractions purified by reversed-phase HPLC containing the peak of CNTF activity were dissolved in 1 percent ammonium bicarbonate and cleaved with Endoprotease Glu-C at 25°C, Endoprotease Asp-N, Endoprotease Lys-C, or chymotrypsin (Boehringer Mannheim) at 37°C overnight at a 1:1 ratio of enzyme to substrate. Peptides were separated on a narrow-bore C8 reversed-phase HPLC column (Applied Biosystems) and sequenced with a gas phase protein sequencer (Applied Biosystems).
8. R. K. Saiki *et al.*, *Science* **239**, 487 (1988). PCR reaction products were subcloned into M13mp10 vectors that had been cut with Sma I and treated with phosphatase. Plaques were screened with γ-^{32}P-labeled oligonucleotides [T. Maniatis, E. F. Fritsch, J. Sambrook, *Molecular Cloning: A Laboratory Manual* (Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, 1982)].
9. Polyadenylated RNA was prepared from rabbit sciatic nerves and liver [A. Ullrich *et al.*, *Science* **196**, 1313 (1977); V. Glisin, R. Crkvenjakov, B. Cyrus, *Biochemistry* **13**, 2633 (1974)]. A sciatic nerve cDNA library in λgt10 phage was constructed with oligo(dT)-primed, double-stranded cDNA [U. Gubler and B. Hoffman, *Gene* **25**, 263 (1983)]. Long, ^{32}P-labeled DNA probes were prepared by random priming [A. P. Feinberg and B. Vogelstein, *Anal. Biochem.* **132**, 6 (1983)]. Hybridization conditions were 5× SSCP (1× SSCP is 20 m*M* sodium phosphate buffer, *p*H 6.8, 15 m*M* sodium citrate, 150 m*M* sodium chloride), 2× Denhardt's, 2 m*M* EDTA, 0.05% sodium pyrophosphate, 0.1% SDS, herring sperm DNA (250 μg/ml), *p*H 8.0, at 65°C; washing at 65°C in 0.1× SSCP and 0.1% SDS. Sequencing was done by the dideoxy chain-termination method [F. Sanger, S. Nicklen, A. R. Coulson, *Proc. Natl. Acad. Sci. U.S.A.* **74**, 5463 (1977)] with the template being single-stranded DNA prepared from subclones in both orientations in M13 vectors.
10. The transient expression vector consisted of a pBR322-based plasmid containing the cytomegalovirus (CMV) immediate early promoter and enhancer [M. Boshart *et al.*, *Cell* **41**, 521 (1985)]. The simian virus 40 (SV40) origin of replication was located upstream from the CMV promoter and the SV40 polyadenylation sequences were located downstream from the CMV promoter [V. B. Reddy *et al.*, *Science* **200**, 494 (1978)]. The 1.5-kb Eco RI restriction fragment of the rabbit cDNA clone was subcloned into the Eco RI site located between the CMV promoter and SV40 polyadenylation sequences in the expression vector. Correctly oriented subclones were selected on the basis of restriction mapping. Plasmid DNA was prepared from expression vector plus cDNA insert or vector alone by alkaline lysis with subsequent CsCl density centrifugation and transfected into nonconfluent COS-7 cell cultures [L. M. Sompayrac and K. J. Danna, *Proc. Natl. Acad. Sci. U.S.A.* **78**, 7575 (1981)].
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18. Peptides A (NKNINLDSVDGVPMA), B (ESYVKHQGLNKN), and C (KIRSDLTALTESYVKH) were synthesized, conjugated to Keyhole Limpet hemocyanin, and emulsified 1:2 with complete Freund's adjuvant. New Zealand White rabbits were injected subcutaneously with 100 μg per rabbit in multiple dorsal sites. Boosters were given at 2- to 3-week intervals and contained 50 μg of conjugated peptide in incomplete Freund's adjuvant. Peptide affinity columns were prepared by conjugating peptide to activated CH–Sepharose 4B (Pharmacia). Immunized rabbit serum was passed over the column, and bound antibodies were eluted as described [E. Harlow and D. Lane, Eds., *Antibodies: A Laboratory Manual* (Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, 1988), pp. 313–315]. The titer of antibodies to the peptides was determined by enzyme-linked immunosorbent assay by using peptide-coated wells [J. A. Tainer *et al.*, *Nature* **312**, 127 (1984)].

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