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Research Article

The Cholinergic Neuronal Differentiation Factor from Heart Cells Is Identical to Leukemia Inhibitory Factor

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A protein secreted by cultured rat heart cells can direct the choice of neurotransmitter phenotype made by cultured rat sympathetic neurons. Structural analysis and biological assays demonstrated that this protein is identical to a protein that regulates the growth and differentiation of embryonic stem cells and myeloid cells, and that stimulates bone remodeling and acute-phase protein synthesis in hepatocytes. This protein has been termed D factor, DIA, DIF, DRF, HSFIII, and LIF. Thus, this cytokine, like IL-6 and TGFβ, regulates growth and differentiation in the embryo and in the adult in many tissues, now including the nervous system.

A GROUP OF PROTEINS, OFTEN CALLED CYTOKINES, REGULATE growth and differentiation in a wide variety of tissues, both in the embryo and in the adult organism. Some of these proteins, such as interleukin-6 (IL-6), were first recognized for their effects on myeloid cells. The generation of the diverse array of myeloid cells is under the control of cytokines and proteins termed

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hematopoietic regulators. The genes for many of these proteins and their receptors have been cloned, and considerable information is available on their biological activities in vitro, as well as some data on their actions in vivo in mouse and man (*1*). In the nervous system, it is also clear that phenotypic decisions can be controlled by factors in the local environment, both in vitro (*2, 3*) and in situ (*4–6*). It has been difficult to characterize these neuronal differentiation factors because the protein concentrations are very low, and the assays are lengthy and utilize nondividing, primary neurons. Nonetheless, a 45-kD glycoprotein that can control neuronal phenotypic decisions has been purified from rat heart cell conditioned medium (*7, 8*). This protein, usually referred to as the cholinergic neuronal differentiation factor, acts on postmitotic, rat sympathetic neurons to specifically induce the expression of acetylcholine (ACh) synthesis and cholinergic function, while suppressing catecholamine (CA) synthesis and noradrenergic function (*7–10*). The protein can also specifically alter neuropeptide gene expression in these neurons (*11*), and it very likely affects the development of other types of peripheral and central neurons as well (*12*). It is termed a differentiation factor because it controls phenotypic choices in these neurons without affecting their survival or growth (*9*).

Using highly sensitive protein sequencing methods, we have obtained amino acid sequence data for seven peptides from the cholinergic factor. An affinity purified antiserum made against a synthetic peptide corresponding to the NH_2-terminal 11 amino acids is able to specifically precipitate the differentiation activity, demonstrating that the sequenced protein is, in fact, the active factor. The amino acid sequences of the seven peptides are nearly identical to the corresponding peptides of the hematopoietic factor that can inhibit the proliferation and induce macrophage differentiation of the leukemic myeloid M1 cell line [leukemia inhibitory factor, LIF (*13*), which is also known as D factor and human macrophage differentiation inducing factor, DIF (*14*)], maintain the developmental potential of embryo carcinoma cells and embryonic stem cells [differentiation inhibitory activity, DIA, and differentiation retarding factor, DRF (*15*)], and support the proliferation of the leukemic DA-1a cell line (HILDA) (*16*), although the latter identification is in doubt (*17*). We used our amino acid sequence data and the available complementary DNA (cDNA) sequence information to clone a cDNA for the rat protein. Comparison of the sequences of the rat cholinergic factor with mouse and human LIF confirms their identity. In biological assays, recombinant murine LIF (recLIF) duplicates the cholinergic differentiation activity.

Protein sequencing of the cholinergic factor and production of antisera. In order to sequence the NH_2-terminal of the cholinergic factor, we collected 40 liters of serum-free conditioned medium from secondary, rat heart cell cultures, and purified the protein (*7*). An estimated 0.5 to 0.8 μg of the 45-kD protein was electroblotted onto an activated glass fiber filter, and sequenced on a Caltech gas phase sequenator at an initial signal of 18 pmol (*18*). Three separate samples yielded an NH_2-terminal, 14–amino acid sequence of EPLPITPVSATXAI (*19*). To minimize the possibility that the 45-kD band containing the cholinergic activity was contaminated by another protein, we isolated the band by SDS–polyacrylamide gel electrophoresis (PAGE); the material in the band was eluted, chemically deglycosylated with anhydrous hydrogen fluoride, purified further by SDS-PAGE, and sequenced (*20*). The chemically deglycosylated protein was approximately the same size, 20 kD, as that obtained after enzymatic deglycosylation with endoglycosidases (*7*). The sequence obtained for the 20-kD band was EPLPITPV, identical to that of the intact protein.

In order to be certain that the sequenced protein was, in fact, the cholinergic factor, we obtained antisera to an 11–amino acid peptide corresponding to the NH_2-terminal sequence (*21*). These antisera

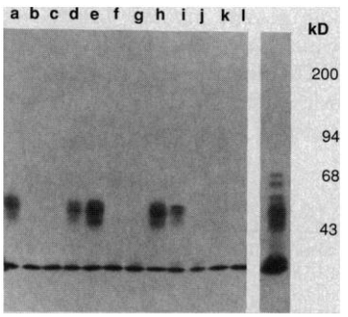

Fig. 1. Precipitation of the 45-kD protein by antisera to a synthetic peptide corresponding to the NH_2-terminal sequence of the protein. Rabbit antisera to the peptide coupled to the carrier proteins KLH (lanes a to d), ovalbumin (lanes e to h), or not coupled to any carrier (lanes i and j). The partially purified factor (Sephadex fraction) was labeled with ^{125}I, and the labeled proteins are shown in the lane next to the molecular sizes. The ^{125}I-labeled proteins were incubated (*21*) with antisera that had been incubated with the following blockers: free peptide (lanes b, f, and j), peptide conjugated to the appropriate carrier proteins (lanes c and g), KLH (lane d), ovalbumin (lane h), or was incubated with antiserum to ovalbumin (lane l) or without any antiserum (lane k). The immune complexes were adsorbed by protein A-Sepharose, washed, eluted, and analyzed by SDS-PAGE. A similar experiment, with affinity-purified antibodies, yielded similar results.

Table 1. Precipitation of cholinergic activity by antibodies to peptide. Immunoprecipitation experiments were performed as in Fig. 1 (*21*), with antibodies purified by peptide affinity-column chromatography. When antibodies were incubated with free peptide, the sample was extensively dialyzed after the incubation to remove free peptide. The immune complexes were centrifuged and proteins were eluted from the pellets by acid treatment. Antibodies could also be eluted by this treatment. However, addition of excess peptide to the eluate before neutralization to block rebinding of the eluted antibodies to the eluted factors resulted in no change in cholingeric activity. Both the eluates from the immune pellets and the immune supernatants (Sup) were added to sympathetic neuronal cultures from days 2 to 16, at 2-day intervals. The ratio of transmitters produced (acetylcholine to catecholamine; ACh/CA) (*7*) is a quantitative measure of the two transmitter systems and reflects, in part, the levels of choline acetyltransferase and tyrosine hydroxylase, respectively (*9*).

Addition to Sephadex fraction	ACh/CA	
	Sup	Eluate
Antibodies to peptide-KLH	0.20	2.40
Antibodies to peptide-KLH plus free peptide	2.13	0.05
Antibodies to KLH	2.64	0.24
No antibodies added	4.46	

(and affinity purified antibodies) could precipitate both the 45-kD protein (Fig. 1) and the cholinergic activity (Table 1) (*22*). Prior incubation of the antisera with the synthetic peptide prevented this precipitation, indicating that the protein of interest had been sequenced. The antisera to the NH_2-terminus recognized the native protein, suggesting that this portion of the protein is exposed on its surface. The precipitates in Fig. 1 also reveal other, weaker bands near the 45-kD protein, and addition of the synthetic peptide abolished the precipitation of all of these bands. Since the 45-kD protein has several glycosyl chains (*7*), the additional bands in the precipitated fractions may be glycosylation variants of this protein or the result of partial proteolytic degradation.

The extremely high degree of degeneracy in the codons for the NH_2-terminal sequence made cloning by oligonucleotide screening difficult; we therefore purified a large amount of the cholinergic factor from 35 liters of conditioned medium (*23*) and determined the sequences of a number of internal peptides (*24*). The following sequences were obtained: LGQGEPFPNNVDKL; SQLAQLPVV; LGASL; LVE; EAFE; LIAT; and EPLPITPV. The fact that one of the peptides corresponded to the same NH_2-terminal sequence already obtained showed that the tryptic peptides came from the same protein. A search of the computer database revealed a close match of each of these peptides with a corresponding peptide in the

murine and human LIF-DIF-D-factor-DIA-DRF protein (*13*), which we refer to as LIF.

Cloning the cDNA for the cholinergic factor and its identity with LIF. To determine the correct sequence for rat cholinergic factor or LIF, we used the polymerase chain reaction (PCR), with oligonucleotides corresponding to the signal peptide and COOH-terminal sequences of murine and human LIF (*25*). The rat, murine, and human amino acid sequences are aligned in Fig. 2, and the corresponding peptides that were sequenced are underlined. Although the COOH-terminus of the rat sequence is not yet complete, there is a strong homology with both the murine (92 percent identity) and the human (82 percent identity) LIF amino acid sequences. It is striking that there are highly nonconservative differences between all three species at residues 103, 113, 146, 150, and 152, and a cysteine residue (at 146) is present only in the rat sequence. The differences between the deduced rat amino acid sequence (Fig. 2) and sequences derived from the tryptic peptides (given above) are found primarily in the NH_2-terminal ends of the peptides and at putative *N*-glycosylation sites. These are sites known to present difficulties for accurate amino acid sequencing.

In order to confirm that the cloned product of the murine LIF (*13*) does indeed have cholinergic neuronal differentiation activity, we obtained an authentic sample of this material (*26*). Purified murine recLIF, expressed in *Escherichia coli* (*27*), was added to cultures of neonatal rat sympathetic neurons, and its effects on the choice of transmitter phenotype were assayed; LIF was able to induce ACh synthesis as well as suppress CA synthesis at low concentrations (Table 2). These are the activities observed for the cholinergic differentiation factor purified from heart cell conditioned medium (*7*).

We have presented several lines of evidence to demonstrate that the proteins previously called LIF and the cholinergic neuronal differentiation factor are the same molecule. The purified neuronal factor and recLIF have the same biological activity when tested with cultured neurons. The slight differences between the amino acid sequences obtained from the tryptic peptides and the deduced sequence for rat LIF can be attributed to limitations of protein sequencing methods. In addition, Gearing *et al.* (*13*) detected only one gene on Southern blots of murine genomic DNA digested with nine restriction endonucleases and probed with a LIF cDNA at both high and low stringencies.

The various names that have been used for this protein may deserve reconsideration. For example, the previous term used with neurons, cholinergic differentiation factor, is inappropriate for two reasons. First, recent results with pure factor and recLIF on cultured sympathetic neurons show that the protein specifically induces the expression of several neuropeptides in addition to the biosynthetic enzyme for acetylcholine (*11*). Second, several reports on the effects of the protein on myeloid cell lines and embryonic stem cells have led to the introduction of at least five different names. Furthermore, the protein has been implicated in bone remodeling and in the induction of acute phase plasma protein synthesis in liver cells (*14, 17, 28*).

Although the role of the cholinergic factor in the normal development of the nervous system is not yet known, the fact that

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rlif ATGAAGGTCTTGGCCGCAGGGATTGTGCCCCTACTGCTC---ATTCTGCAC 48
mlif ATGAAGGTCTTGGCCGCAGGGATTGTGCCCT       GCTGG
RLIF                                LeuLeuLeu---IleLeuHis -7
MLIF MetLysValLeuAlaAlaGlyIleValPro          LeuVal
HLIF                        Val              ---Val

                          ++1
rlif TGGAAACACGGGGCAGGGAGCCCCCTTCCCATCACCCCTGTAAATGCCACC 99
mlif                         T
RLIF TrpLysHisGlyAlaGlySerProLeuProIleThrProValAsnAlaThr 11
MLIF
HLIF

rlif TGCGCCATACGCCACCCGTGTCACGGCAACCTCATGAACCAGATCAAGAGT 150
mlif   T             A C                              A
RLIF CysAlaIleArgHisProCysHisGlyAsnLeuMetAsnGlnIleLysSer 28
MLIF                                                  Asn
HLIF                          Asn                  ArgSer

rlif CAACTGGCTCAACTCAACGGCAGTGCCAATGCCCTCTTTATTTCCTATTAC 201
mlif        A G     T     C        T     C
RLIF GlnLeuAlaGlnLeuAsnGlySerAlaAsnAlaLeuPheIleSerTyrTyr 45
MLIF
HLIF                                             Leu

rlif ACAGCTCAAGGGGAACCATTTCCCAACAACGTGGATAAGCTATGTGCGCCA 252
mlif           A G G                A              T
RLIF ThrAlaGlnGlyGluProPheProAsnAsnValAspLysLeuCysAlaPro 62
MLIF                                 Glu
HLIF                               Leu             Gly

rlif AACATGACGGATTTCCCACCTTTCCATGCCAATGGGACAGAGAAGACCAAG 303
mlif        A C      T        G  C
RLIF AsnMetThrAspPheProProPheHisAlaAsnGlyThrGluLysThrLys 79
MLIF                 Ser      Gly
HLIF   Val                                          Ala

rlif TTGGTCGAGCTGTATCGGATGGTCACGTACCTGGGAGCCTCCCTGACCAAC 354
mlif     G                   A     A C              T
RLIF LeuValGluLeuTyrArgMetValThrTyrLeuGlyAlaSerLeuThrAsn 96
MLIF                        Ala     Ser
HLIF                   Ile   Val         Thr      Gly

rlif ATCACCTGGGATCAGAAAAACCTCAACCCCACTGCCGTGAGCCTCCAGATC 405
mlif       C    C     GGT   G                        G
RLIF IleThrTrpAspGlnLysAsnLeuAsnProThrAlaValSerLeuGlnIle 113
MLIF       Arg         Val                           Val
HLIF       Arg         Ile          Ser   Leu      HisSer

rlif AAACTCAATGCGACTACAGACGTCATGAGGGGGCTCCTTAGCAGCGTGCTT 456
mlif   G        T    T               C     C    AT
RLIF LysLeuAsnAlaThrThrAspValMetArgGlyLeuLeuSerSerValLeu 130
MLIF                Ile                          Asn
HLIF                Ala   IleLeu                 Asn

rlif TGCCGTCTGTGCAACAAGTACCATGTGGGCCATGTGGATGTGCCCTGTGTC 507
mlif                        G          C           ACC
RLIF CysArgLeuCysAsnLysTyrHisValGlyHisValAspValProCysVal 147
MLIF                        Arg                     Pro
HLIF             Ser                              ThrTyrGly

rlif CCCGACAACTCTAGCAAAGAAGCCTTCCAAAGCAAGAAGTTGGGCTGCCAG 558
mlif       C     GA                          A       T
RLIF ProAspAsnSerSerLysGluAlaPheGlnArgLysLysLeuGlyCysGln 164
MLIF       His   Asp
HLIF       Thr   Gly   AspVal      Lys

rlif CTCCTGGGGACATACAAGCAAGTCATAAGTGTGTTGGCCCAGGCCTTCTAG 609
mlif   T                   GTCATAAGTGTGGTGGTCCAGGCCTTCTAG
RLIF LeuLeuGlyThrTyrLysGln                          180
MLIF                       ValIleSerValValValGlnAlaPheTER
HLIF           Lys          Ile   Ala   LeuAla         TER
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Fig. 2. The nucleotide sequence of the coding region of rat LIF (*rlif*) and its deduced amino acid sequence (RLIF) are compared to those of mouse (*mlif*, MLIF) and human (HLIF), which have been described earlier (*13*). The suggested NH_2-terminal residue is designated as +1 with an arrow. MLIF has an additional amino acid in the signal sequence compared to the rat and human sequences; this difference is indicated by the dashed lines. Serine is suggested to be the NH_2-terminal residue because our NH_2-terminal amino acid sequencing data yielded one residue before proline, and Gly-Ser is a likely signal cleavage site. The regions of the deduced protein that correspond to the peptides obtained in our amino acid sequencing experiments are underlined. The first and last 30 nucleotides correspond to the primers used for the PCR reactions and were taken from the mouse or human sequences.

Table 2. Cholinergic differentiation activity of LIF. Sympathetic neurons were grown for 2 weeks in the presence of 100 percent volume equivalents of heart cell conditioned medium, recombinant (rec) murine LIF, or no additions other than the usual medium constituents. The concentration of cholinergic factor protein in 100 percent heart cell conditioned medium is estimated to be between 10 and 100 ng/ml. Transmitter synthesis from radioactive precursors was assayed as described (*7*); the data are expressed as means ± SEM, with $n = 4$. The ACh/CA values are the means of the ratios from individual cultures. ACh, acetylcholine; CA, catecholamine.

Material tested	Activity per neuron (fmol/hour)		
	ACh	CA	ACh/CA
Control	0.04 ± 0.01	2.70 ± 0.41	0.02 ± 0.01
recLIF (1 ng/ml)	0.20 ± 0.04	1.10 ± 0.23	0.29 ± 0.13
recLIF (10 ng/ml)	1.30 ± 0.02	0.50 ± 0.08	2.70 ± 0.45
Heart cell conditioned medium	3.20 ±1.25	0.52 ± 0.16	6.60 ± 0.35

sympathetic neurons [and possibly sensory and spinal cord neurons as well (*12*)] show a striking and specific response to it suggests that they have receptors for the protein as well as a compatible second messenger system. It is important to ascertain where these receptors are localized during neuronal development. Although there is good evidence for the localized production of a phenotype-specifying factor by a target tissue in situ (*6*), all types of neurons that innervate a given target do not respond similarly to that environment. Thus, the known diversity of neuronal phenotypes could be due to different combinations of instructive cues present at various locations and to differential responsiveness to a given mixture of cues at any one site. The availability of the appropriate genes, proteins, and antisera should lead to clarification of the similarities and differences among the various factors that have been reported to influence neuronal phenotype. This includes not only the cholinergic factors (*29*), but other neuronal differentiation activities as well (*30*), because the cholinergic factor itself can turn on (and off) the expression of several different neurotransmitters and neuropeptides (*11*).

If the same molecule influences the differentiation of myeloid, neural, and other cells, how then is the specificity of its control on each of these different systems maintained? Side effects, if any, could be averted if the distribution of the factor is highly localized, or if the various systems are responsive during different developmental periods. There are other examples of proteins that are potentially important for the development of both the immune and nervous systems. Interleukin-1 (IL-1) and possibly IL-3, and receptors for IL-1, -2, and -4 are found in the brain and in neural cell lines (*31, 32*). IL-1 stimulates glial proliferation, somatostatin, and nerve growth factor (NGF) production, and it has transmitter-like effects on neurons (*33*). IL-2 stimulates oligodendroglial proliferation and maturation as well as corticotropin release from pituitary cells (*32*). Gamma-interferon can enhance astrocyte maturation and indirectly increase the cholinergic differentiation of cultured spinal cord neurons, and rat interferon enhances the expression of acetylcholine receptors in cultured rat myotubes (*34*). B cell stimulatory factor 2 (or IL-6) is detected in glial cell lines and can induce neurite extension and voltage-dependent sodium channels in PC12 cells (*35*). Conditioned medium from activated lymphocytes can maintain sympathetic neurons in culture, and brain macrophages can release NGF in vitro (*36*). NGF can promote hemopoietic colony growth and differentiation, and injection of NGF into neonatal rats increases the number of mast cells (*37*). In addition, subsets of thymocytes and lymphocytes express NGF receptors (*37*). In fact, there are many parallels in the questions and phenomenology of lineage decisions in the neural crest and hematopoietic systems (*38*). Our data add a new dimension to this overlap; very different lineage choices may be controlled by the same molecules.

Note added in proof: We have determined the 3′ sequence of the rat cDNA, and the final 30 bases are GTCATTAGTGCGGTGGTCCAGGCCTTCTAG.

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18. A small fraction of the factor preparation was labeled with ^{125}I and added to the unlabeled protein as a tracer. For the final step in the purification, the sample was run on a 12.5 percent SDS-polyacrylamide gel (0.7 mm thick) and electroblotted onto an activated glass fiber filter. Whatman GF/F glass fiber filters were covalently derivatized with trimethoxysilypropyl-*N*,*N*,*N*-trimethylammonium chloride after etching the glass with trifluoracetic acid as described (R. H. Aebersold, D. B. Teplow, L. E. Hood, S. B. H. Kent, *J. Biol. Chem.* **261**, 4229 (1986)). Electroblotting was carried out in 25 m*M* tris, 192 m*M* glycine, 0.5 m*M* dithiothreitol (DDT), *p*H 8.3 at 50 V for 2 hours at 10°C. The filter was air dried at room temperature and the proteins were visualized by autoradiography. Alternatively, the blot was stained with the fluorescent dye, 3,3′-dipentyloxacarbocyanine iodide, and bands were visualized by illumination at 254 nm. The region of the filter at 45 kD was cut out with a razor blade and inserted into the cartridge of a sequenator. Phenylthiohydantoin (PTH) derivatives from each cleavage were then analyzed [M. W. Hunkapiller and L. E. Hood [*Methods Enzymol.* **91**, 486 (1983)] on an IBM cyano column, except that 5 to 7 percent (v/v) tetrahydrofuran was added to buffer A and the *p*H was adjusted to 5.1.
19. Abbreviations for the amino acid residues are: 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; Y, Tyr; and X, undetermined.
20. For chemical deglycosylation, the gel-eluted protein was extensively dried; redistilled HF (100 μl) was added and the reaction was allowed to proceed on ice for 30 minutes. The HF was evaporated under a stream of nitrogen, residual HF was neutralized with 100 m*M* $(NH_4)HCO_3$, and the sample was dried at reduced pressure. The protein was dissolved in SDS-PAGE sample buffer for the final electrophoresis.
21. The peptide was synthesized and coupled to the carrier proteins keyhole limpet hemocyanin (KLH) and ovalbumin by standard methods. The free peptide and the peptide conjugates were diluted to 1 mg/ml in PBS (phosphate-buffered saline), *p*H 7.5, and mixed with an equal volume of complete Freund's adjuvant. Each peptide (1 mg) was injected into each of three New Zealand White rabbits (6 to 10 weeks old), subcutaneously. After 1 month, the animals were injected with 0.5 mg of antigen in incomplete Freund's adjuvant, and bled 10 days after each injection. Antibody titers were determined by a solid phase radioimmunoassay, with ^{125}I-labeled protein A for detection of bound antibody. The antibodies to the peptides were purified on a peptide affinity column. Long-chain alkylamine-controlled pore glass (Sigma) (1 g) was activated with 2 mg of maleimidobenzoyl *N*-hydroxysuccinimide ester in dimethylformamide and coupled with 3 mg of reduced peptide in 100 m*M* sodium phosphate (*p*H 7.0) at 30°C for 1 hour. Unreacted groups were blocked by excess DTT, and the beads washed and equilibrated with PBS. The antiserum or its protein A-Sepharose purified immunoglobulins were incubated in batches with the column material for 3 to 15 hours and washed twice with 20 ml of PBS, *p*H 7, then with 9 ml of PBS containing 0.5 percent Tween and 1*M* NaCl, followed by 100 ml of PBS. Specifically bound antibodies were eluted with 18 ml of 0.1*M* glycine-HCl, *p*H 2.5. The eluate was promptly neutralized with 1*M* tris-HCl, *p*H 8, and concentrated by Centricon 30 (Amicon). The antibodies were dialyzed against PBS, frozen, and stored. The ratio of antibodies to the peptide to

antibodies to the carrier was >10:1, on the basis of binding curves with the peptide and the carrier proteins as antigens.
22. Partially purified cholinergic factor was iodinated with the Bolton-Hunter reagent (7) and used as a tracer. The Sephadex fraction, containing 2×10^5 cpm of tracer, was first incubated with preimmune serum (overnight at 4°C) in the presence of protease inhibitors [1 m*M* phenylmethylsulfonyl fluoride (PMSF), and Trasylol, leupeptin, and turkey egg white trypsin inhibitor at 2 μg/ml each]. The complexes that bound to preimmune serum were absorbed by protein A-Sepharose in PBS, 0.02 percent azide, 0.01 percent polyethylene glycol (PEG) for 2 hours, and were removed by centrifugation. The supernatant was then incubated overnight at 4°C with antiserum to the peptides (which had been incubated overnight at 4°C with or without blockers) in the presence of protease inhibitors and reprecipitated. The protein A-Sepharose bearing immune complexes were washed once with PBS, 0.02 percent azide, 0.01 percent PEG, four times with high salt buffer (0.1*M* tris-HCl, *p*H 7.8, 0.5*M* NaCl, 0.02 percent azide, and 0.01 percent PEG), and then with PBS, azide, and PEG. Immune complexes were eluted by 0.1*M* glycine-HCl, *p*H 2.5, 0.01 percent PEG and neutralized promptly with 1*M* tris-HCl, *p*H 8. These results were reported in an abstract [K. Fukada, *Soc. Neurosci. Abstr.* **12**, 106.5 (1986)].
23. The cholinergic factor was purified as described (7), except that the precipitation with 60 percent ammonium sulfate was omitted. The purification was monitored by precipitation of the iodinated factor (which had been added to the preparation) with the antiserum to the factor. This procedure works well only for the CM-cellulose and Sephadex fractions.
24. The preparation and sequencing of internal peptides followed the protocol of Aebersold *et al.* [R. H. Aebersold, J. Leavitt, R. A. Saavedra, L. E. Hood, S. B. H. Kent, *Proc. Natl. Acad. Sci. U.S.A.* **84**, 6970 (1987)]. Partially purified cholinergic factor (2 to 3 μg) was separated from contaminating protein by SDS-PAGE. The proteins were transferred to nitrocellulose, and the band corresponding to the cholinergic factor was digested with trypsin on the nitrocellulose. The resulting peptides were released into the supernatant and separated by high-performance liquid chromatography (HPLC). The most prominent peptides were then sequenced.
25. Four oligomers were used to obtain the PCR fragment of rat LIF: (i) a 21-nucleotide (nt) oligomer (ATGAAGGTCTTGGCCGCAGGG) starting from the initiation codon of murine LIF; (ii) 20 nt (CTAGAAGGCCTGG$^{G}_{A}$CCA$^{C}_{A}$CA) starting from the antisense sequence of the stop condon of the murine and human factor; (iii) 30 nt (ATGAAGGTCTTGGCCGCAGGGATTGTGCCC) corresponding to the 5′ end of the signal peptide of murine LIF; (iv) 30 nt (CTAGAAGGCCTGG$^{G}_{G}$CCA$^{A}_{C}$CACACTTATGAC) corresponding to the COOH-terminus of the protein. Complementary DNA was synthesized from polyadenylated cultured heart cell RNA with a MuLV (murine leukemia virus) reverse transcriptase kit (BRL) and oligomer (ii) as the primer. During the first 30 cycles of the PCR reaction [94°C for 1 minute, 50°C for 2 minutes, and 72°C for 3 minutes; R. K. Saiki *et al.*, *Science* **239**, 487 (1988)] the oligomers of (i) and (ii) were the primers. The first PCR product of approximately 600 bp was purified and used as the template of the second PCR reaction with oligomers of (iii) and (iv). The second PCR product contained a major band of the expected size (609 bp) in agarose gel electrophoresis. The fragment was subcloned to the vector of PGEM-3zf(−) (Promega), and analyzed with the Sequenase (U.S. Biochemical) system. Both strands were sequenced not only with the T7 and SP6 primers, but also with oligomers (iii) and (iv) and three internal sequences (TTTTTCTGATCCCAGGTGAT, TTCCCCTTGAGCTGTGTAATAGGAA, GAGAAGACCAAGTTGGTCGAGCTGTA).
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39. We thank D. Metcalf and colleagues at the Walter and Eliza Hall Institute for Medical Research in Melbourne for the recLIF; M. Tanouye and M. Ramaswami for help with the PCR DNA amplification; T. Hunkapiller and D. Jackson for help with the computer searches; D. Anderson, H. Nawa, and M. Rao for advice, D. McDowell for help with the preparation of tissue culture materials; J. Carnahan for NGF, J. Frost, M. Ryder, and R. Pittman for conditioned medium, S. Horvath and colleagues for oligonucleotide and peptide synthesis, B. Jentoft-Nilsen and M. Aranda for help with nucleic acid sequencing, and S. Ou for help with antibodies. Supported by NINCDS (Javits Neuroscience Investigator Award), a McKnight Foundation Neuroscience Research Project Award, a Lucille P. Markey Charitable Trust Developmental Biology grant, the Gustavus and Louise Pfeiffer Research Foundation, and the Joseph Drown Foundation (P.H.P.); by the Muscular Dystrophy Association and Dysautonomia Foundation (K.F.); by the American Heart Association and the Otto Hahn Medaille (S.K.); by the Alzheimer's Disease and Related Disorders Association (T.Y.); a Swiss National Foundation Fellowship (R.A.); and the Ministry of Education, Taiwan, R.O.C., and the Helen G. and Arthur McCallum Foundation (M.-J.F.).

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