The Receptor for Ciliary Neurotrophic Factor

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Although neurotrophic factors were originally isolated on the basis of their ability to support the survival of neurons, these molecules are now thought to influence many aspects of the development and maintenance of the nervous system. Identifying the receptors for these neurotrophic factors should aid in identifying the cells on which these factors act and in understanding their precise mechanisms of action. A "tagged-ligand panning" procedure was used to clone a receptor for ciliary neurotrophic factor (CNTF). This receptor is expressed exclusively within the nervous system and skeletal muscle. The CNTF receptor has a structure unrelated to the receptors utilized by the nerve growth factor family of neurotrophic molecules, but instead is most homologous to the receptor for a cytokine, interleukin-6. This similarity suggestes that the CNTF receptor, like the interleukin-6 receptor, requires a second, signal-transducing component. In contrast to all known receptors, the CNTF receptor is anchored to cell membranes by a glycosyl-phosphatidylinositol linkage.

THE DEVELOPMENT AND FUNCTION OF THE NERVOUS SYSTEM depends on proteins, termed neurotrophic factors, originally defined by their ability to support the survival of neuronal cells (*1*). In addition to promoting neuronal survival, these factors can influence proliferative and differentiative processes within the nervous system and may also have actions outside of the nervous system (*2*). Much has been learned about the receptors for the prototypical neuronal survival molecule, nerve growth factor (NGF), as well as its two structural relatives, brain-derived neurotrophic factor (BDNF) and neurotrophin-3 (NT-3) (*3*). The product of the *trk* proto-oncogene, which is mainly expressed in the nervous system and which structurally resembles a receptor tyrosine kinase, constitutes a crucial component of the NGF receptor (*4*). The *trk*B gene encodes a protein that is structurally related to the protein product of *trk*, but that binds and mediates functional responses to both BDNF and NT-3 (*5*). The role of a second receptor component, which binds all three NGF-related factors (*5, 6*), designated the low-affinity NGF receptor (LNGFR) because it was initially defined by its ability to bind NGF with low affinity, remains unclear (*4, 7*). The utilization of receptor tyrosine kinases by the family of NGF-related factors is consistent with reports that NGF activates tyrosine phosphorylation, as well as intermediate kinases that depend on tyrosine phosphorylation, in responsive cells (*8*).

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In contrast, little is known about the receptor for ciliary neurotrophic factor (CNTF), a fourth neurotrophic agent that has no structural homology to NGF, BDNF, and NT-3 (*9*). Although CNTF was isolated on the basis of its ability to support the survival of embryonic chick ciliary ganglion neurons in vitro (*9, 10*), it also promotes the survival of sympathetic and sensory neurons in vitro and motoneurons in vivo (*10, 11*). Furthermore, CNTF induces cholinergic differentiation of cultured rat neurons (*12*), and promotes differentiation of glial progenitor cells into type 2 astrocytes (*13*). We have described a method for the detection of cells bearing CNTF receptors that is based on binding assays utilizing a genetically engineered CNTF molecule that contains an epitope tag at its carboxyl terminus and have identified several neuronal cell lines that bind and respond to CNTF (*14*).

Using one of these cell lines and the tagged CNTF in a tagged-ligand panning procedure, we have cloned a receptor for CNTF. The structure of this receptor is unrelated to the receptors utilized by the NGF family of neurotrophic molecules, but instead is similar to the receptor for the cytokine interleukin-6 (IL-6).

Isolation of complementary DNA clones encoding the CNTF receptor. The modified rat CNTF contained a 10–amino acid extension at its carboxyl terminus that was derived from the human c-*myc* proto-oncogene product (*14*). This modified CNTF-myc allowed the identification of CNTF receptors with the use of the tagged ligand as a bridge between the receptor and a murine monoclonal antibody [9E10 (*15*); designated in this article as α-myc], which was specific for an epitope in the c-myc tag. We used this tagged CNTF (CNTF-myc) in an expression cloning strategy that was based on a modification of the panning procedure of Aruffo and Seed (*16*).

The human neuroblastoma cell line SH-SY5Y, which binds CNTF and displays a functional response to CNTF, was our source of messenger RNA for constructing a library in a mammalian expression vector, pCMX (*17*). This library was transfected into COS cells to begin the first cycle of tagged-ligand panning (Fig. 1A). Transfected cells were incubated sequentially with CNTF-myc and α-myc, and then added to plates coated with a secondary antibody that bound α-myc. This procedure enriched for cells that expressed a CNTF receptor, because such cells would adhere to the plate by means of an indirect bridge consisting of CNTF-myc and α-myc. Cells that did not adhere were washed off the plates; plasmid DNA from the remaining cells was reintroduced into COS cells for another cycle of tagged-ligand panning.

We assessed the enrichment for plasmids encoding the CNTF receptor by assaying the transfectants for CNTF binding. This was accomplished by incubating cell monolayers sequentially with CNTF-myc, α-myc, and a ^{125}I-labeled secondary antibody against α-myc. Autoradiography of the plates treated in this manner revealed individual transfected COS cells expressing a CNTF receptor. The first round of panning demonstrated substantial enrichment: although only a few receptor-positive cells were detected in COS cells transfected with the unenriched library, hundreds of receptor-

positive cells were evident in COS cells transfected with the enriched library (Fig. 1B). After a second round of panning, individual plasmid clones were isolated and assayed for their ability to elicit CNTF-myc binding when transfected into COS cells. Of 15 plasmid clones tested, 14 were positive by this indirect binding assay. Restriction analysis of the 14 putative cDNA clones for the CNTF receptor revealed four distinct classes of DNA inserts, although they all shared an identical overlapping region.

The expression of CNTF binding activity in transfected cells was assessed further by flow cytometry with a secondary antibody coupled to fluorescein isothiocyanate to detect bound CNTF-myc. As judged by this analysis, the specific binding of CNTF-myc to COS cells that had been transfected by a positive receptor clone was 100 times greater than the background binding (Fig. 1C).

Cross-linking the CNTF receptor with ^{125}I-labeled CNTF. To identify polypeptides encoded by the CNTF receptor cDNA clones, we carried out chemical cross-linking with ^{125}I-labeled CNTF. Analysis of the cross-linked products by gel electrophoresis revealed a prominent labeled species of approximately 97 kD in COS cells transfected with the putative CNTF receptor cDNA, but not in control COS cells; the appearance of this cross-linked species was eliminated by excess unlabeled CNTF (Fig. 2A). Similar cross-linking studies with ^{125}I-labeled CNTF were carried out with a cell line that responds functionally to CNTF [the human neuroblastoma cell line (SH-SY5Y) from which the receptor cDNA was obtained] as well as with CNTF-responsive neurons [sympathetic neurons from rat superior cervical ganglia (SCG)]. In both cases a major, specific cross-linked species was observed that was indistinguishable in size from that observed with COS cells transfected with the cloned receptor cDNA (Fig. 2B). Because the molecular mass of CNTF is approximately 25 kD, the CNTF-binding protein expressed on transfected COS cells, on SH-SY5Y cells, and on sympathetic neurons must have a molecular mass of approximately 72 kD. Other, less prominent cross-linked species, including a faint heterogeneous species of about 150 to 200 kD were also observed.

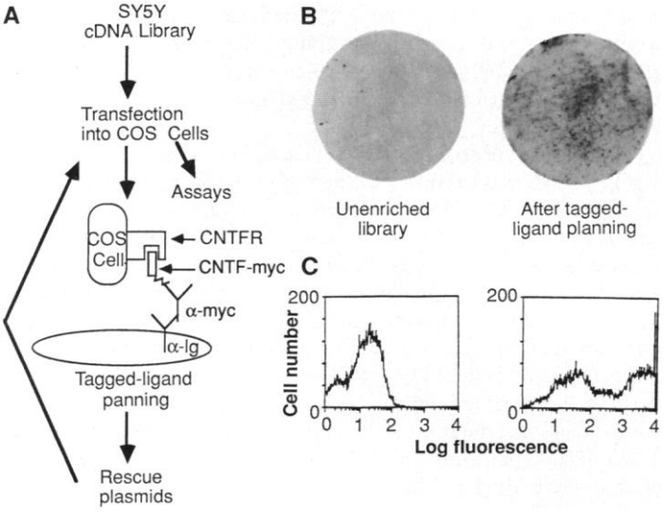

Fig. 1. Expression cloning of the CNTF receptor by tagged-ligand panning. (**A**) The strategy used to enrich for CNTF receptor cDNA. (**B**) Autoradiographs of plates transfected with equal amounts of unenriched (left) or enriched (right) library and then assayed by incubating successively with CNTF-myc, α-myc, and ^{125}I-labeled goat antibody to mouse immunoglobulin G. (**C**) Flow cytometric analysis of COS cells that were transfected with either a negative clone (left) or with the CNTF receptor (right). Cells were incubated with CNTF-myc, α-myc, and fluorescein isothiocyanate (FITC)–conjugated goat antibody to mouse immunoglobulin G before analysis with a fluorescence-activated cell sorter (FACS).

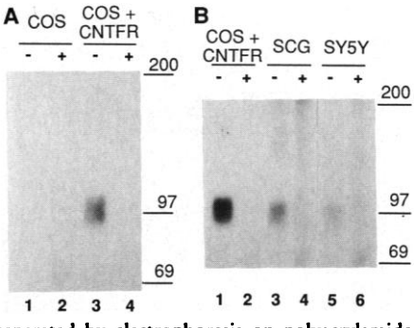

Fig. 2. ^{125}I-labeled CNTF cross-linked to proteins in neuroblastoma cells, primary neurons, and COS cells transfected with CNTF receptor cDNA. Cross-linking was performed with the bifunctional cross-linking reagent, disuccinimidyl suberate (DSS), in the presence (+) or absence (−) of 1 μM unlabeled CNTF. Cross-linked species were separated by electrophoresis on polyacrylamide gels and visualized by autoradiography. (**A**) Cross-linking of ^{125}I-labeled CNTF to untransfected COS cells or COS cells transfected with the CNTF receptor, as described (*5*). (**B**) Cross-linking of ^{125}I-labeled CNTF to COS cells transfected with the CNTF receptor or to crude membrane preparations isolated from SHSY5Y cells or SCG's (*32*).

These may represent oligomerized receptors, distinct receptors, or other components of a receptor complex (see below).

Sequence of the CNTF receptor. Sequence analysis revealed that the four distinct classes cDNA clones encoding the CNTF receptor all contained an identical long open reading frame of 372 amino acids (Fig. 3A). In vitro translation of these cDNA clones verified that they encoded a protein of approximately 41 kD (*18*). This observation supported our assignment of the presumed open reading frame and indicated that substantial post-translational modification of this protein must occur to result in the 72-kD receptor defined by the cross-linking experiments. In fact, *N*-glycanase treatment reduced the apparent size of the CNTF receptor by about 20 kD, suggesting that several of the four potential glycosylation sites are utilized in the native molecule (*19*). The remaining difference in apparent molecular mass may be accounted for by other post-translational modifications (see below).

Searches of databases revealed that the predicted amino acid sequence of the CNTF receptor most closely resembles that of the IL-6 receptor. Like the extracellular portion of the IL-6 receptor (*20, 21*), the CNTF receptor could be divided into two domains; the IL-6 and CNTF receptors share approximately 30 percent sequence identity within these two domains. Within the first, immunoglobulin-like domain (*20*), both the CNTF and IL-6 receptors are homologous to several members of the immunoglobulin superfamily, including carcinoembryonic antigen (CEA), the PDGF receptor, the receptor for colony stimulating factor–1 (CSF-1), and the α-1-β–glycoprotein (Figs. 4A and 5). After this immunoglobulin-like domain, both the CNTF and IL-6 receptors contain a segment that shares structural features with an extracellular domain found in a superfamily of receptors (*21, 22*) for a variety of peptide hormones (growth hormone and prolactin), interleukins (IL-3, IL-4, IL-5, IL-6, and IL-7), and other hematopoietic factors (G-CSF, GM-CSF, and erythropoietin) (Figs. 4B and 5). This cytokine receptor-like domain includes four conserved cysteine residues and the sequence motif WSXWS near the carboxyl terminal; the second components of some multisubunit receptors also contain these motifs (namely, the receptors for IL-2, CM-CSF, and IL-6) (Fig. 5).

The sequence similarities between the CNTF and IL-6 receptors do not extend past the cytokine receptor–like domain. After this domain, the IL-6 receptor possesses a well-defined transmembrane region, a stop-transfer signal, and a short cytoplasmic domain of approximately 100 amino acids (*23*). Hydropathy analysis revealed that the CNTF receptor contains a hydrophobic region at its amino terminus, which appears to be typical of secretory signal peptides, as well as a hydrophobic region after the cytokine receptor–like

domain that is located at the carboxyl terminus of the protein (Fig. 3B). Although this latter hydrophobic region is in a position analogous to the transmembrane domain of the IL-6 receptor, it is not followed by a stop-transfer signal nor an intracytoplasmic domain. Thus, unlike its closest relative, the CNTF receptor completely lacks a cytoplasmic domain. It is interesting that the IL-6 receptor does not require its intracellular domain in order to mediate signal transduction (*23*).

Membrane anchoring of the CNTFR by a glycosyl-phosphatidylinositol linkage. The absence of a cytoplasmic domain in the sequence of the CNTF receptor was reminiscent of certain other cell surface proteins that lack intracellular domains. Examples of such proteins include Thy-1, CEA, the variant surface glycoproteins (VSG) of *Trypanosoma brucei*, N-CAM, and the scrapie prion (*24*). In all of these proteins, the hydrophobic regions that are present in the cDNA sequences at their carboxyl termini are absent from the mature proteins. These regions are cleaved off during processing, and the proteins are anchored to the cell surface by a glycosyl-phosphatidylinositol (GPI) linkage (*24*). In general, the GPI linkage is sensitive to treatment with phosphatidylinositol-specific phospholipase C (PI-PLC) (*25*).

We tested whether the CNTF receptor is also anchored by a GPI linkage by determining whether PI-PLC could release the CNTF receptor from the SH-SY5Y cell line, from SCG-derived sympathetic neurons, and from COS cells transfected with the cloned receptor cDNA. In all of these cells, the specific binding of ^{125}I-labeled CNTF was reduced by more than 50 percent after PI-PLC treatment (Fig. 6A). In control experiments, PI-PLC had no effect on the binding of ^{125}I-labeled NGF to the low-affinity NGF receptor expressed on A875 human melanoma cells.

To demonstrate that the receptor was released intact from the cell surface by PI-PLC, a tagged version of the receptor was constructed in which the c-myc epitope was incorporated near the amino terminus of the receptor. This allowed identification of the receptor on Western blots with α-myc. The tagged CNTF receptor was transfected into COS cells, which were then treated with PI-PLC. Cells and supernatants were then subjected to Western (immunoblot) analysis. PI-PLC treatment substantially reduced the amount of CNTF receptor remaining on the cells (Fig. 6B, lanes 3 and 4) and released a large amount of the receptor into the supernatant (Fig. 6B, lanes 5 and 6); the electrophoretic mobility of the released receptor was larger than that of the membrane bound form.

PI-PLC cleavage of GPI anchors exposes an antigenic epitope, termed CRD (cross reacting determinant) (*26*), which is localized in the glycan region of the GPI anchor or the released protein. Antibodies to CRD recognize the cleaved anchors of many GPI-anchored proteins (*24*). To determine whether this epitope was present in tagged CNTF receptor released form cells by PI-PLC, supernatants from PI-PLC–treated COS cell transfectants were immunoprecipitated with α-myc and then analyzed by Western blotting with antibody to CRD as a probe. Antibodies to CRD recognized the PI-PLC–released protein, but not the uncleaved protein (Fig. 6B, lanes 7 to 10), indicating that the CNTF receptor that had been cleaved with PI-PLC displayed the CRD determinant. Taken together, these results demonstrate that the CNTF receptor is bound to the cell surface by means of a GPI linkage.

CNTF receptor expression in the nervous system and in skeletal muscle. To begin to define normal cell populations potentially responsive to CNTF, Northern blot analysis was performed on RNA samples from a variety of rat tissues. A probe derived from the coding region of the CNTF receptor identified a 2-kb transcript that was expressed mainly in the central nervous system (Fig. 7). Within the brain the CNTF receptor transcript was detectable in all regions examined but in different amounts. The cerebellum had the largest

Fig. 3. (**A**) Nucleotide sequence and predicted amino acid sequence of the CNTF receptor cDNA. Sequencing was performed with the dideoxy chain termination method, with Sequenase (U.S. Biochemicals). Bars indicate hydrophobic regions of the sequence. Potential *N*-glycosylation sites (Asn-X-Ser/Thr) are boxed. The sequence has been deposited with Genbank (accession number M73238). (**B**) Hydropathy plot of the CNTF receptor amino acid sequence. The plot was generated with the MacVector sequence analysis program (International Biotechnologies), with a window length of 19. Larger numbers on the *y*-axis indicate a higher degree of hydrophilicity.

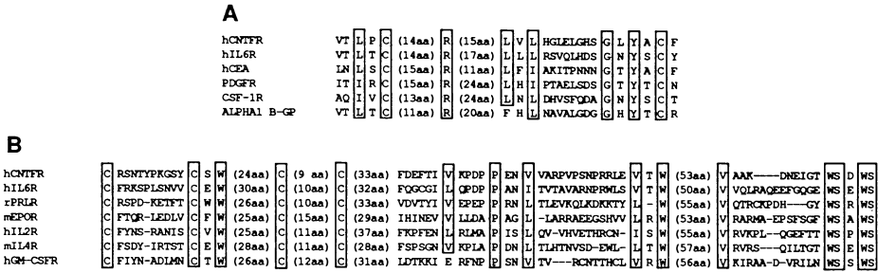

Fig. 4. Comparison of the sequence of the CNTF receptor with other known sequences. Representative sequences were aligned by inspection, with gaps (indicated by dashes) introduced to improve the alignments. Numbers in parentheses indicate residues that were omitted for the sake of brevity. Boxes enclose residues that are highly conserved among the indicated sequences. (**A**) Immunoglobulin-like domain. hCEA, human carcinoembryonic antigen; PDGFR, platelet derived growth factor receptor; CSF-1R, colony-stimulating factor–1 receptor; ALPHA1 B-GP, α-1-B–glycoprotein. (**B**) Cytokine receptor–like domain. PRLR, prolactin receptor; EPOR, erythropoeitin receptor; IL2R, interleukin-2 receptor (β chain); IL4R, interleukin-4 receptor; GM-CSFR, granulocyte-macrophage–stimulating factor receptor. 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; and Y, Tyr.

amount of transcript (Fig. 7). Outside of the central nervous system, low amounts of CNTF receptor expression could be detected in the adrenal gland and sciatic nerve (*19*). High expression of CNTF receptor mRNA was also found in a single non-neural normal tissue, skeletal muscle (Fig. 7). In a large panel of cell lines, CNTF receptor expression was limited to neuronal and muscle lines (*19*).

Comparisons of the receptor systems. The cDNA clones we have obtained encode a polypeptide that is indistinguishable in size from the endogenous CNTF-binding receptor expressed in a CNTF-responsive human cell line and in primary rat sympathetic neurons. Furthermore, the cloned receptor shares with its endogenous counterparts the unusual property of GPI linkage to the cell surface. These observations indicate that the cloned receptor is closely related to, or identical with, the receptor normally utilized by CNTF-responsive cells. The expression cloning of the CNTF receptor illustrates the utility of the tagged-ligand panning approach for receptor cloning. In principle, any new ligand may be modified in a similar way in order to clone its receptor, provided that the addition of an antigenic peptide tag to the ligand does not interfere with stable binding to the receptor and that the tag present on receptor-bound ligand remains accessible to an antibody.

The resemblance of the CNTF receptor to the IL-6 receptor provides potential insights into the mechanism of action of CNTF. Although the actual mechanism of signal transduction triggered by the binding of IL-6 to its receptor is not yet known, a second transmembrane protein that is required for IL-6 signal transduction has been identified (*23*). The existence of such a signal transducer was predicted on the basis of the small size of the intracytoplasmic portion of the IL-6 receptor, as well as on the finding that the deletion of this region does not interfere with signal transduction (*23*). The IL-6 signal transducer, gp130, has been cloned (*27*). Although gp130 itself does not bind IL-6, association between gp130 and the IL-6 receptor is triggered by the binding of IL-6 to its receptor; this association apparently results in high-affinity IL-6 binding. The extracellular domain of gp130 resembles that of the G-CSF receptor and consists of an amino-terminal cytokine receptor–like domain, followed by a domain with some homology to contactin, a cell surface protein (Fig. 5). The intracellular domain of gp130 contains motifs for guanosine triphosphate (GTP) binding but bears little resemblance to known GTP-binding proteins; gp130-mediated signal transduction apparently does not proceed by any of the pathways known to be activated by cytokines or growth factors (*28*).

The absence of a cytoplasmic domain in the CNTF receptor and its homology with the IL-6 receptor suggest that the CNTF receptor requires a second cell surface component to mediate a functional response. Because gp130 is expressed in many cells that lack IL-6 receptors and do not respond to IL-6 (*27*), CNTF might utilize gp130 as its second component; alternatively, the CNTF signal transducer may be a molecule with functional properties resembling those of gp130.

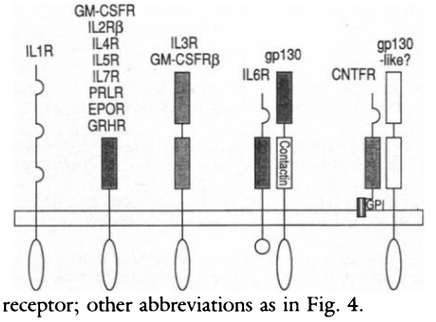

Fig. 5. Structural comparison of the CNTF receptor with other known receptors. Half-circle, the immunoglobulin-like domain described in Fig. 4A; shaded box, cytokine receptor–like domain described in Fig. 4B; large ovals, possible signal transducing cytoplasmic domains; striped box, GPI anchor. GRHR, growth hormone receptor; other abbreviations as in Fig. 4.

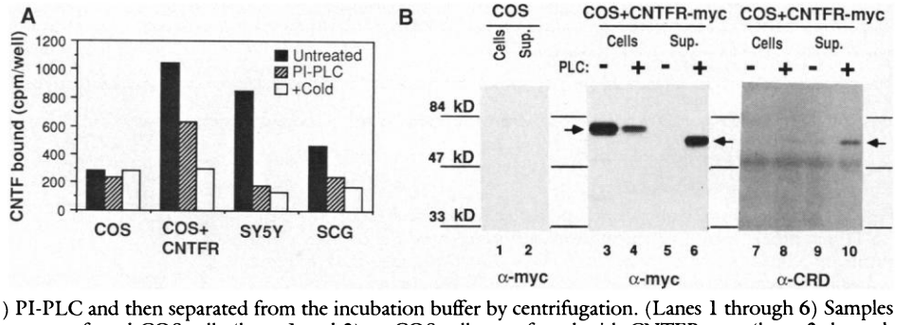

Fig. 6. GPI-linkage characteristics of the CNTF receptor. (**A**) Effect of PI-PLC treatment on CNTF binding by untransfected COS cells, COS cells transfected with the CNTF receptor cDNA clone, SH-SY5Y cells, and primary cultures of rat SCGs. Cell monolayers were incubated in the presence (PI-PLC) or absence (untreated) of enzyme. ^{125}I-labeled CNTF (100 pM) with (+ Cold) or without 100 nM unlabeled CNTF was then added, and bound radioactivity was determined. (**B**) Western blot analysis of PI-PLC release of the CNTF receptor from cell membranes and exposure of the CRD epitope. Cells were treated in suspension with (+) or without (−) PI-PLC and then separated from the incubation buffer by centrifugation. (Lanes 1 through 6) Samples (either solubilized cells pellets or supernatant) from untransfected COS cells (lanes 1 and 2) or COS cells transfected with CNTFR-myc (lanes 3 through 6) were subjected to electrophoresis on polyacrylamide gels, transferred to membranes, and probed with α-myc. (Lanes 7 through 10) Samples (solubilized cells or supernatant from COS cells transfected with CNTF receptor-myc) were incubated with α-myc and then bound to agarose beads conjugated with goat antibodies to mouse immunoglobulin G. The resulting immunoprecipitates were blotted with antibodies to CRD.

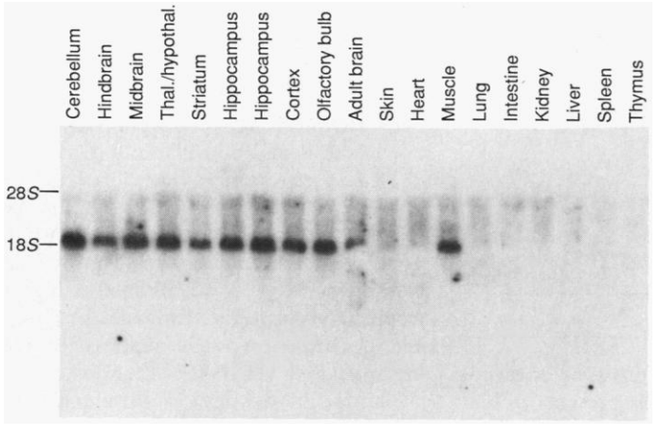

Fig. 7. Expression of the CNTF receptor in adult rat tissues. Total RNA was isolated from the indicated tissues (*33*). RNA was fractionated on 1 percent formaldehyde–agarose gels and transferred to nylon membranes. Blots were hybridized with a ^{32}P-labeled probe derived by amplification with the polymerase chain reaction from CNTF receptor cDNA (*33*). Thal./hypothal., thalamus or hypothalamus (or both). The hippocampal samples were independent.

In contrast to the IL-6 receptor as well as to all other known receptors, the CNTF receptor is anchored to the cell surface by means of a GPI linkage. There may be situations in which activation of an endogenous phospholipase might release the receptor, providing a mechanism for regulating the cellular response to CNTF. The released receptor might act to absorb free CNTF. Alternatively, a complex of CNTF and the released receptor might mediate functional responses in cells that do not express the receptor but do express the signal transducing component. In fact, a secreted form of the IL-6 receptor can associate with gp130 and mediate functional responses to IL-6 (*27*). The observation that the CNTF receptor is GPI-linked suggests that other GPI-linked proteins, although not generally considered to be capable of transmembrane signaling, might transmit signals indirectly by second components.

Our findings reveal that the receptor system employed by CNTF differs from the receptor tyrosine kinases utilized by the NGF family of neurotrophic factors; thus, although neurotrophic activities may appear superficially similar, their precise actions might be quite different. The CNTF receptor system appears to be related to a very large family of cytokine receptors that mediate a wide array of pleiotropic effects throughout the body. Cytokine receptor–like molecules may also play diverse roles in the nervous system; the effects of IL-6 on neuronal cells (*29*), the variety of actions attributed to CNTF, as well as the number of CNTF-like activities that have been described (*30*), would be consistent with this notion (*31*).

A relatively restricted set of neurons and glial precursor cells have been shown to display functional responses to CNTF. The widespread expression of the CNTF receptor in neural tissue suggests that additional cells throughout the nervous system may be capable of responding to this protein. The expression of the CNTF receptor in skeletal muscle similarly suggests an unanticipated role for CNTF in muscle biology.

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