

Gene transfer and molecular cloning of the rat nerve growth factor receptor

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The amino-acid sequence of one form of the receptor for nerve growth factor defines a new class of growth factor receptors.

ALTHOUGH nerve growth factor (NGF) was the first growth factor to be described¹ and its physiologically relevant flow from target tissue to neuronal cell body is well characterized², much less is known about the transduction of signals from the NGF receptor (NGFR) or the intracellular pathways involved in mediating the functions of NGF than is the case for many mitogenic growth factors, neurotransmitters or hormones. It is known that NGF, which is required for the development of sympathetic and some sensory neurons as well as their viability in adulthood^{2,3}, is secreted by the target tissue near the nerve terminal, binds to a receptor and is internalized by receptor-mediated endocytosis^{4,5}. The internalized NGF-containing vesicles are retrogradely transported along microtubules to the cell body⁶. Although the NGF is delivered intact and biologically active to the cell body it is quite rapidly degraded in the lysosomes^{7,8}. Somewhere in this pathway the intracellular signals that are key to functions of NGF are generated.

A better understanding of what these signals are and how they are generated is likely to follow from further knowledge of the receptor or receptors for NGF. Two apparent types of NGFR exist on the NGF-responsive neurons and in PC12 cells. The major population has a lower affinity for NGF ($K_d \sim 1$ nM) and releases NGF rapidly after binding, compared to the minor population whose affinity is two orders of magnitude higher and which release NGF relatively slowly⁹. For these reasons the NGFR are identified as the fast (low-affinity) NGFR and slow (high-affinity) NGFR. Occupied slow NGFR are trypsin resistant and insoluble in Triton X-100, whereas occupied fast NGFR are trypsin labile and soluble in Triton X-100⁹. Agents which cluster the receptor, such as wheat germ agglutinin¹⁰⁻¹² or antibodies to NGF¹², convert the fast type to a slow type of NGFR, suggesting a relationship between the types of NGFR¹³. The slow and fast NGFR when crosslinked with ¹²⁵I-NGF using *N*-hydroxysuccinimidyl-4-azido-benzoate (HSAB) form complexes of relative molecular mass 158,000 and 100,000 (M_r 158K and 100K) respectively, the latter containing a single receptor peptide chain¹⁴. Internalization of NGF in PC12 cells uses only the slow NGFR^{15,16} but both types of NGFR may be involved in this process in sympathetic neurons *in vivo*¹⁷.

To explore the relationship between the two NGFR further and with the ultimate aim of identifying receptor-mediated intracellular signals, the complementary DNA for the fast NGFR from the rat PC12 cells has been cloned. A two-step approach was used. First, using genomic-DNA-mediated gene transfer, a set of mouse cell lines that express the rat fast NGFR were produced. A cDNA clone that coded for the rat fast NGFR was then isolated based on the difference in gene expression in the newly created NGFR⁺ cell lines and the original mouse cell line. The 426 amino-acid sequence deduced from the cDNA sequence shows that the fast NGFR is unrelated to any other growth factor receptor. Indirect evidence suggests that the slow NGFR comprises the fast NGFR moiety together with a second subunit.

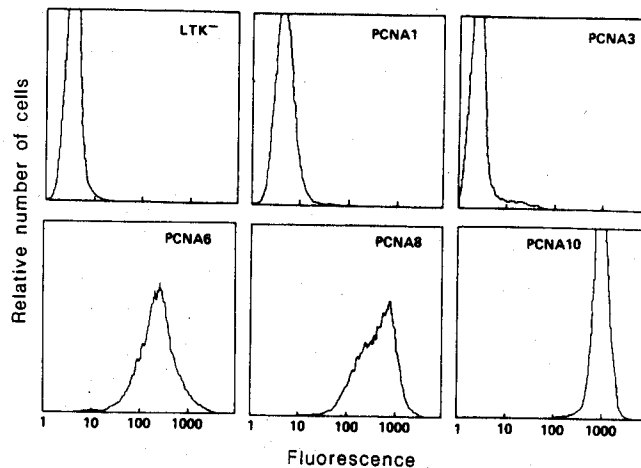


Fig. 1 Amplification of the expression of NGFR in the PCNA cell line. The first panel shows a control staining of LTK⁻ cells. The remaining panels show the amplification of NGFR expression in the PCNA cells after repeated rounds of sorting. The number attached to the cell line name indicates the number of times the cells had been sorted.

Methods. LTK⁻ cells (10^6) were transfected with 20 μ g of PC12 genomic DNA and 1 μ g of pCHTK using a modified CaPO₄ procedure of Wigler *et al.*^{18,19}. Stable transfectants were selected by growth in hypoxanthine, aminopterin and thymidine (HAT) medium¹⁹. After 2-3 weeks of HAT selection the transfectants were harvested in phosphate-buffered saline (PBS) with 1 mM EDTA. Cells (10^6) were then collected by centrifugation and resuspended in 50 μ l of staining medium¹⁹ containing 1 μ g of the anti-NGFR monoclonal antibody, MC192 (ref. 20). After 30 min of incubation at 4 °C the cells were washed once with 200 μ l of staining medium and resuspended in 50 μ l of 4 °C staining medium plus 1 μ g of fluorescein-conjugated goat anti-mouse IgG and IgM (Tago, Inc.). After 20 min an equal volume of staining medium (2 ng propidium iodide ml⁻¹) was added to detect dead cells. Ten minutes later the cells were washed twice with 200 μ l of staining medium. The stained cells were then resuspended in 200 μ l of staining medium, passed through a 50 μ m screen and sorted on a FACS. The most intensely staining cells (~1%) were collected and grown for 2-3 weeks in HAT medium at which time there were enough cells to stain and sort again.

Transfection and amplification

Mouse LTK⁻ cells were cotransfected with high-molecular-mass rat PC12 pheochromocytoma genomic DNA (100-200 kilobases (kb) in length) and plasmid (pCHTK) containing the chicken thymidine kinase gene^{18,19}, and LTK⁺ cells selected by growth in HAT medium¹⁸. To detect transfectants that expressed the rat fast NGFR the LTK⁺ cells were stained with MC192, a mouse monoclonal antibody against the fast NGFR²⁰, and fluorescein-conjugated goat anti-mouse immunoglobulin G

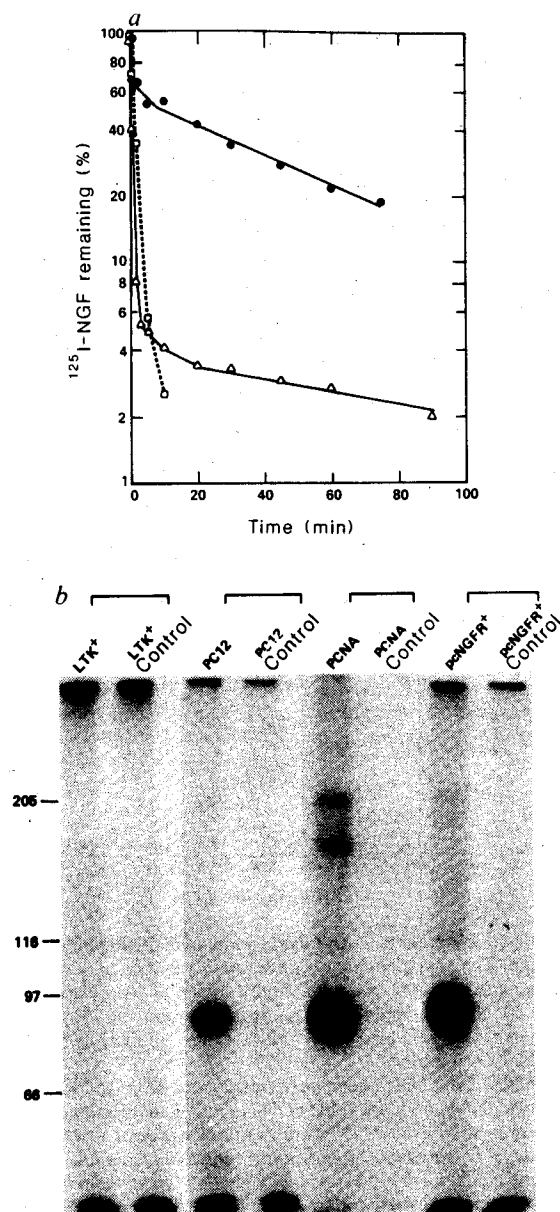


Fig. 2 Characterization of the NGFR in PC12 and transfected cell lines. *a*, Dissociation kinetics of PC12 (●), PCNA (Δ) and pcNGFR⁺ (□) cells. Results are expressed as the percentage of the initial specific binding before dissociation. PCNA and pcNGFR⁺ cells were washed once, harvested in Ca²⁺ Mg²⁺-free PBS, 1 mM EDTA, pH 7.4, and washed in 10 mM HEPES/Krebs-Ringer saline, pH 7.4 (Krebs-Ringer), containing 1 mg ml⁻¹ each of glucose and bovine serum albumin (binding buffer). PC12 cells were harvested by trituration and washed four times in binding buffer. PC12 cells (2 × 10⁶ ml⁻¹), PCNA cells (0.5 × 10⁶ ml⁻¹), and pcNGFR⁺ cells (2 × 10⁶ ml⁻¹) were incubated with 100 pM [¹²⁵I]-NGF in 1.5 ml of binding buffer for 60 min at 22 °C, dissociation initiated by addition of 500–1000-fold unlabelled NGF at 37 °C, and residual binding assayed⁹. *b*, Determination of the size (*M_r*) of the NGFR. Lanes 1, 3, 5 and 7 are analyses of immunoprecipitates obtained with the MC192 antibody from LTK⁺, PC12, PCNA, and pcNGFR⁺ cell surfaces respectively. Control lanes are of immunoprecipitates with anti-agrin. The cells were harvested as described above, except that glucose and BSA were omitted. LTK⁺ cells (12 × 10⁶), PC12 cells (14 × 10⁶), PCNA cells (13 × 10⁶) and pcNGFR⁺ cells (11 × 10⁶) were surface radiiodinated⁴² and solubilized in 1 ml of 0.5% Nonidet P40 Krebs-Ringer containing a mixture of 10 protease inhibitors. After one hour on ice, nuclei were removed by centrifugation at 4 °C. To one half of each supernatant 5–6 μg of anti-receptor MC192 antibody was added and the suspension was incubated at 4 °C with mixing for 12 h. The remainder (controls) received 10 μg of antibody raised against agrin (gift of Dr U. J. McMahan). After centrifugation at 10,000g for 10 min, 50 μl of a suspension of goat anti-mouse antibodies coupled to agarose were added to each supernatant and incubated at 4 °C with mixing for an additional 12 h. Immunoprecipitates were collected and washed four times with 0.5% NP40. After removal of excess NP40, immunoprecipitates were stored at -20 °C until analysed by SDS-polyacrylamide gel electrophoresis.⁴³

(IgG). Positive cells were then isolated using a fluorescence-activated cell sorter (FACS).

In eight independent transfections, six positive cell lines were obtained. The fluorescence intensity of five of them was similar to that of PC12 cells. The sixth cell line had a heterogeneous, unstable phenotype. With repeated growth in HAT medium and sorting on the FACS the average intensity continued to increase. After 10 rounds of sorting and regrowth, a stable cell line, PCNA, was obtained (Fig. 1).

Analysis of the transfected L cell lines

The transfectants were analysed by several methods to ensure that they expressed PC12 NGFR and to determine the form of receptor. The rate of dissociation of [¹²⁵I]-NGF from PCNA cells showed that it expressed only fast NGFR (Fig. 2*a*). Similar results were obtained with all the other transfectants. Scatchard analysis of the steady-state binding of PCNA cells revealed a single class of NGFR with a *K_d* (2.3 nM) identical to that of PC12 fast NGFR, and a number of NGFR (2.3 × 10⁶ per cell) more than 10-fold greater than on PC12 cells. Analysis of the immunoprecipitates of [¹²⁵I]-labelled PC12 and PCNA cell-surface proteins with the MC192 antibody showed the same

major protein of *M_r* 83K (Fig. 2*b*), the expected size of the fast NGFR^{14,21}. Although not apparent in this figure, the immunoprecipitate actually comprises two species with *M_r*s of 82K and 85K. The minor 200K species observed in these analyses is probably a disulphide-linked oligomer of the 83K species^{22,23}. Finally, only the 100 K crosslinked, fast NGFR-NGF complex was observed in PCNA cells after crosslinking with HSAB. From these data we conclude that transfer of the rat fast NGFR into mouse L cells was achieved.

Cloning of the rat fast NGFR cDNA

The rat fast NGFR gene was rescued by relying on the fact that the NGFR⁺ L-cell transfectants express fast NGFR mRNA whereas the L cells do not. In brief, a fast-NGFR-enriched probe was made by subtracting ³²P-labelled PCNA cDNA (made, in turn, from PCNA poly(A)⁺ RNA) with a 20-fold excess of LTK⁺ poly(A)⁺ RNA²⁴ to remove constitutive LTK⁺ cDNA. The remaining ³²P-labelled cDNA (10% of total cDNA) was used to screen 60,000 colonies of a PCNA cDNA library in pUC9²⁵⁻²⁷. The expression of the fast NGFR is very high in PCNA cells, so it seemed likely that the cDNA for this receptor would be one of the more abundant species detected with the subtracted

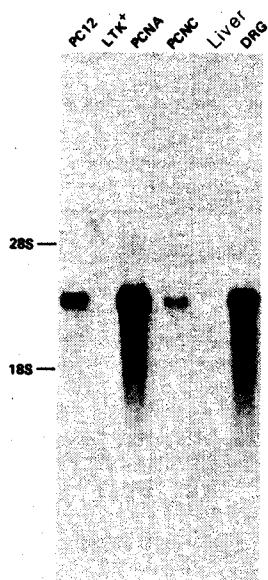


Fig. 3 Northern analysis of the NGFR cDNA clone. Total RNA⁴⁴ (10 μ g) from PC12 cells, LTK⁺ cells, PCNA cells, PCNC cells, rat liver and newborn rat dorsal root ganglia (DRG) were each separated on a 0.8% formaldehyde agarose gel⁴⁵ and blotted to a nylon membrane according to the manufacturer's instructions (Hybond, Amersham Corp.) A ³²P-labelled cDNA probe ($\sim 30 \times 10^6$ c.p.m.) was made from the putative NGFR cDNA clone insert (pNGFR.1), using random primers⁴⁶ and hybridized with the blot in 10 ml of 50% formamide, 5 \times SSPE, 5 \times Denhardt's, 1 mM ATP and 0.1% SDS for 3 days at 42°C. The blot was then washed 4 times for 30 min in 0.2 \times SSC and 0.1% SDS at 65°C and subjected to autoradiography²⁵.

probe. Based on this assumption 30 of the most intensely hybridizing colonies were picked. Rescreening reduced this number to 19. To simplify the identification of fast NGFR clones the 19 clones were separated into groups based on cross-hybridization of their inserts. From one family of clones that reacted most strongly with the subtracted probe, a clone with an insert of 3.4 kb was selected. This insert detected a common 3.7-kb message in PC12, PCNA and PCNC (another transfectant) cells and sensory (dorsal root ganglion) neurons which express fast NGFR, but not in LTK⁺ or rat liver cells which do not (Fig. 3), suggesting that it contained a fast NGFR cDNA.

The 3.4-kb clone was inserted into the simian virus 40(SV40)-based vector, pcDL1, in sense and antisense directions and the two subclones (pcNGFR⁺ and pcNGFR⁻) independently transfected into LTK⁻ cells. The FACS analysis of both LTK⁺ cells and the transfectant with pcNGFR⁻ after HAT selection exhibited background levels of staining, whereas the transfectants with pcNGFR⁺ stained positively. The brightest 10% of these cells were collected, grown in HAT medium and analysed for fast NGFR in the same way as were the genomic DNA transfectants. Scatchard analysis showed a single class of receptors with a K_d of 3.5 nM, similar to that of fast NGFR on PC12 and PCNA cells, and $\sim 17,000$ receptors per cell. The rate of ¹²⁵I-NGF dissociation was also characteristic of the PC12 fast NGFR (Fig. 2a) and immunoprecipitation of ¹²⁵I-labelled surface proteins showed the 83K species, confirming that the fast NGFR in these L cells transfected with pcNGFR⁺ is of the correct size (Fig. 2b). We conclude that the 3.4-kb clone, now identified as pNGFR.1, contains the rat fast NGFR cDNA.

Nucleotide sequence of fast NGFR cDNA

The nucleotide sequence of the fast NGFR cDNA, with its predicted amino-acid sequence, is shown in Fig. 5a. A model of the fast NGFR protein and a restriction map of the pNGFR.1

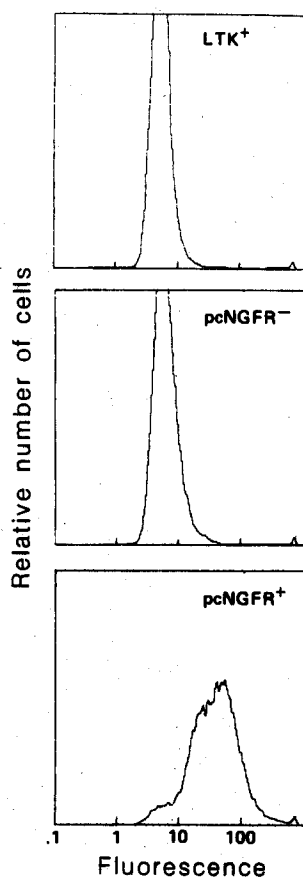


Fig. 4 Transfection of the full length NGFR cDNA in an expression vector into mouse L cells. The complete insert from the putative NGFR cDNA clone, pNGFR.1, was excised with *Eco*RI and *Hind*III and blunt-ended with the large fragment of *Escherichia coli* DNA Pol I²⁵. The insert was then cloned in either orientation in front of the SV40 early gene promoter of pcDL1 at the *Eco*RI site which was also blunt-ended. Plasmid pcDL1, which was made from the Okayama and Berg cDNA cloning vectors pcDV1 and pcL1, was a gift of Dr Frank Lee (DNAX). An *Eco*RI site was inserted into the normal cDNA cloning site. The pcDL1-NGFR constructs (1 μ g), pcNGFR⁺ and pcNGFR⁻, were independently transfected with 1 μ g of pCHTK and 20 μ g of carrier DNA (isolated from A875 cells) into LTK⁻ cells^{18,19}. LTK⁻ cells transfected with A875 genomic DNA and pCHTK served as a control. After HAT selection the stable transfectants were stained with MC192 and FITC conjugated goat anti-mouse IgG and IgM¹⁹. The stained cells were then analysed using the FACS.

insert are given in Fig. 6. The first start codon is 114 bases from the 5' end. The open reading frame continues for 1,275 bases which results in a precursor peptide with M_r 45,432. Preliminary amino-acid sequencing of the fast NGFR, purified from the PCNA cells, indicates that 29 N-terminal amino-acid residues are removed, leaving a lysine at the amino terminus of the mature receptor protein (T.P.M., M.J.R. & E.M.S., manuscript in preparation). The NGFR expressed by the human melanoma cell line, A875 has a nearly identical N-terminal sequence²⁸. Cleavage of this signal peptide results in a mature peptide containing 396 amino-acid residues with M_r 42,478.

Analysis of the hydrophathy of the predicted fast NGFR peptide²⁹ suggests only one plasma membrane spanning domain (residues 225-249). The amino-terminal, probably extracellular, domain of the protein, comprising 222 amino-acid residues, is very rich in cysteine residues, has 2 putative sites for N-linked glycosylation³⁰ and is very acidic. It contains four repeating elements in which the positions of the cysteine residues are highly conserved (Fig. 5b). The intracellular domain of 151

a

1 CAGCTCCGGCGGCGCAGCGCTGGAGCGCATGCGAGTTCAGCTCAGCGCAGCACCATCGGTCTCGGGAGCGGACTGAGCTAGAAAGCGGAGCGCTGACGCCGGAGGCGTGA
 -20 -10 -1
 114 Met Arg Arg Ala Gly Ala Ala [Cys] Ser Ala Met Asp Arg Leu Arg Leu Leu Leu Leu Ile Leu Gly Val Ser Ser Gly Gly Ala Lys
 ATG AGG AGG GCA GGT GCT GCC TGC AGC GCC ATG GAC CGG CTG CGC CTG CTG CTG ATT CTA GGG GTG TCC TCT GGA GGT GCC AAG
 10 20 30
 204 Glu Thr [Cys] Ser Thr Gly Leu Tyr Thr His Ser Gly Glu [Cys] Lys Ala [Cys] Asn Leu Gly Glu Gly Val Ala Gln Pro [Cys] Gly Ala
 GAG ACA TGT TCC ACA GGC CTG TAC ACC CAC AGC GGA GAG TGC TGC AAA GCC TGC AAC TTG GGC GAA TGC GGC CAC CCG TCC GGA GCC
 40 50 60
 294 Asn Gln Thr Val [Cys] Glu Pro [Cys] Leu Asp Asn Val Thr Phe Ser Asp Val Val Ser Ala Thr Glu Pro [Cys] Lys Pro [Cys] Thr Glu [Cys]
 AAC CAG ACC GTG TGT GAA CCC TGC CTG GAC AAT GTT ACA TTC TCC GAT GTG GTG AGC GCC ACT GAG CCG TGC AAG CCG TGC ACC GAG TGC
 70 80 90
 384 Leu Gly Leu Gln Ser Met Ser Ala Pro [Cys] Val Glu Ala Asp Asp Ala Val [Cys] Arg [Cys] Ala Tyr Gly Tyr Tyr Gln Asp Glu Thr
 CTG GGC CTG CAG AGC ATG TCC GCT CCC TGT GTG Glu GAA GAC GAT GCA GTG TGC AGA TGT GCC TAT GGC TAC TAC CAG GAG GAG ACT
 100 110 120
 474 Gly His [Cys] Glu Ala [Cys] Ser Val [Cys] Glu Val Gly Ser Gly Leu Val Phe Ser [Cys] Gln Asp Lys Gln Asn Thr Val [Cys] Glu Glu [Cys]
 GGC CAC TGT GAG GCT TGC AGC GTG TGC GAG GTG GGC TGC GGA CTC GTG TTC TCC TGC CAG GAC AAA CAG AAC ACA GTG TGT GAA GAG TGC
 130 140 150
 564 Pro Glu Gly Thr Tyr Ser Asp Glu Ala Asn His Val Asp Pro [Cys] Leu Pro [Cys] Thr Val [Cys] Glu Asp Thr Glu Arg Gln Leu Arg Glu
 CCA GAG GGC ACA TAC TCA GAA GCC AAC CAC GTG GAC CCG TGC CTA GCC TGC ACG GTG TGC GAG GAC ACT GAG CCG CAG TTA GCG GAG
 160 170 180
 654 [Cys] Thr Pro Trp Ala Asp Ala Glu [Cys] Glu Glu Ile Pro Gly Arg Trp Ile Pro Arg Ser Thr Pro Glu Gly Ser Asp Ser Thr Ala
 TGC ACG CCC TGG GCT GAT GCT GAA TGC GAA GAG ATC CCT GGT CGA TGG ATC CCA AGG TCT ACG CCC CCG GAG GGC TCC GAC AGC ACA GCG
 190 200 210
 744 Pro Ser Thr Gln Glu Pro Glu Val Pro Pro Glu Gln Asp Leu Val Pro Ser Thr Val Ala Asp Met Val Thr Thr Val Met Gly Ser Ser
 CCC AGC ACC CAG GAG CCT GAG GTT CCT CCA GAG CAA GAC CTT GTA CCC AGT ACA GTG CCG GAT ATG GTG ACC ACT GTG ATG GGC AGC TCC
 220 230 240
 834 Gln Pro Val Val Thr Arg Gly Thr Thr Asp Asn Leu Ile Pro Val Tyr [Cys] Ser Ile Leu Ala Ala Val Val Gly Leu Val Ala Tyr
 CAG COT GTA GTG ACC CGC GGC ACC ACC GAC AAC CTC ATT COT GTC TAT TGC TCC ATC TTG GCT GCT GTG GTC GGC CTT GTG GGC TAT
 250 260 270
 924 Ile Ala Phe Lys Arg Trp Asn Ser [Cys] Lys Gln Asn Lys Gln Gly Ala Asn Ser Arg Pro Val Asn Gln Thr Pro Pro Pro Glu Gly Glu
 ATT GGT TTC AAG AGG TGG AAC AGC TGC AAA CAA AAT AAA CAA GGC GCC AAC AGC CGC CCC GTG AAC CAG ACG CCC CCA CCG GAG GGA GAG
 280 290 300
 1014 Lys Leu His Ser Asp Ser Gly Ile Ser Val Asp Ser Gln Ser Leu His Asp Gln Gln Thr His Thr Gln Thr Ala Ser Gly Gln Ala Leu
 AAA CTG CAC AGC GAC ATC TCT GTG GAC AGC CAG AGC CTG CAC CAG CAG ACC CAT ACG CAG ACT GCC TCA GGC CAG CAG CCG CTC
 310 320 330
 1104 Lys Gly Asp Gly Asn Leu Tyr Ser Ser Leu Pro Leu Thr Lys Arg Glu Glu Val Glu Lys Leu Leu Asn Gly Asp Thr Trp Arg His Leu
 AAG GGT GAT GGC AAC CTC TAC AGT AGC CTG CCC CTG ACC AAG CGT GAG GAG GTA GAG AAA CTG CTC AAC GGG GAT ACC TGG CGA CAT CTG
 340 350 360
 1194 Ala Gly Glu Leu Gly Tyr Gln Pro Glu His Ile Asp Ser Phe Thr His Glu Ala [Cys] Pro Val Arg Ala Leu Leu Ala Ser Trp Gly Ala
 GCA GGC GAG CTG GCT TAC CAG CCT GAA CAT ATA GAC TCC TTT ACC CAC GAG GCC TGC CCA GTG CGA GCC CTG GGC AGC TGG GGT GCC
 370 380 390
 1284 Gln Asp Ser Ala Thr Leu Asp Ala Leu Leu Ala Ala Leu Arg Arg Ile Gln Arg Ala Asp Ile Val Glu Ser Leu [Cys] Ser Thr Thr
 CAG GAC AGT GCA ACG CTT GAT GCC CTT TTA GCC GCC CTG CGA CGC ATC CAG AGA GCT GAC ATT GTG GAG AGT CTA TGC AGC GAG TCC ACT
 4390
 1374 Ala Thr Ser Pro Val
 GCC ACA TCC CCA GTG TGA ACTCACAGACTGGAGCCCTGTCTCTCCACATTCGGACAGCATGATGTTCTAGCCAGCCCCACAGAGCTGCCCTCTCCCTCGGGGATGGC
 1487 CCAACGGTCAGAACCGGAGCATCTCTGTGCAAGGGCTCTGTGTCCCACTCTGACTCCGTGCTCCCGAGGGGGCCCTTGCTTCTGACCAACCTCTCTCAGCAAGAGAGAGAG
 1606 AGGACCCACCGAGCTGACTTGTCTCCATTCCTCATCCAGCCCTTCTCTCTTACACATATAGCTGTGTCAGATCTGGGGTGTGACACTAGGAGAAGGGAGCGGGGCCACCCCTAA
 1725 GACTCAGGAGTACTGAAGAACCAGGACCTGGAACCTCCACACTGTGAACCGGAGCAAGGGGGGGGCAATGTTGGTAGGCTAGACCTTCTTCCCTCTCCCTCTGGCCA
 1844 AAGAAGGAGTACCGACCTATCTGAGCTGAAGCAGGTTTGAAGCCAGCCACACTTCTCTCACACACAGGATGGTAAACCAGAGAAAGGACGGGACTGACCTAGGGCCACCA
 1963 ACCACAGGAAGAACAATGAAGGCTGATACACTCCGTTTCTGAATGAGGCTGAAGTGTCTTGTGACAGGGATGGCGTACTTTCAGGGAAATATCTGGAAAGCCATGTCTGCCCGC
 2082 CCCTCAACCACTTCCAGGCCCTACCAACCTTGTGCAGTGAAGTGTGTTGTAAGGGCTGGTCCATGGTCTATTCTGATGGAGTCAAGCTAAGGGCTCAGGCTTATCCATAAGGC
 2230 ATTTTGGAGAGATGAATCTGTAGTGGCTCATTCTTGGCATAAGCTGAAGCCAAACCGGCCCTTAATGTACAGCCCTCGGGGTCAAGAACCAAGGACTCCACCCCAATCCAACA
 2320 CTACTACTACATTACAGATATCTTGTCTTCCCATGGCTCTTTGGGGCTGAGACTAGATCTCTGCTGGGATC
 2439 ACTGCCAGTGAAGATCCGGAGGGGACAGAGCTGAGCTTCACTGGGGCTGTCTTCTCGCCCCGGGTTGCGAGCCCAAGAAATGACTGCATCTGAGCTGGTGTCTGTCTCCAAATGGCC
 2558 TGTGGCAGTCTTCTCTCAGCTTCAAGGCCCTGCAAGGACGATTTCTGAGCAGCCGCGAGGAGGGGCAAGAGGTTTCGGTTCAGTGGCGCTTCTCCCGGCTCTGTGGCTGTT
 2677 TGTGGCAGTCTTCTCTCAGCTTCAAGGCCCTGCAAGGACGATTTCTGAGCAGCCGCGAGGAGGGGCAAGAGGTTTCGGTTCAGTGGCGCTTCTCCCGGCTCTGTGGCTGTT
 2796 TGTGGCAGTCTTCTCTCAGCTTCAAGGCCCTGCAAGGACGATTTCTGAGCAGCCGCGAGGAGGGGCAAGAGGTTTCGGTTCAGTGGCGCTTCTCCCGGCTCTGTGGCTGTT
 2915 AAGACCCAGGAGCATTGTACGCATACCGGGGTGATTTTTATGACCCCAATCTGCAATTCAGACACCTGGGAAGTGGGACATTTTGTGTATTTATTTCTCCCGGAGGCT
 3034 GGGGAGTGGTGGGGGCTCAGGTACGGTTTGTGTTGTTCTGGGGTCTCTCCAGCCTTGTGGGGCAAGTGGAACTCTGGCCCTCCAGCTGGTACTATGAATCTCA
 3153 GACCCCTTCGTGCTCCCGAGCGCTTCCCTTGATCTGTGTAACTTCTGTGGGCCCTCCCAAACTACACATAAAACATACAGGAGGACCAATTAATTGGCAAAAAA

b

11-47 E S G E C - - C K A C N L G E G V A Q P C G A N Q - T V C E P C L D N V T F S D
 51-89 A T E P C K P C T E C L G L Q S M S A P C V E A D D A V C - R C A Y G Y Y Q D E
 90-129 E T G H C E A C S V C E V G S G L V F S C Q D K Q N F V C E E C P E G T Y S D E
 132-171 E V D P C L P C T V C E D T E R Q L R E C T P W A D A E C E E I P G R W I P R S

Fig. 5 a, Nucleic acid sequence of pNGFR.1 cDNA and the predicted amino-acid sequence of the rat fast NGFR. The nucleotides are numbered on the left and the amino-acid residues are numbered above the sequence. Arrows, two possible sites of asparagine-linked glycosylation; round-ended box, putative membrane-spanning domain. The cysteine residues are in rectangles and the four repeating elements are underlined. The sequence was obtained on both strands primarily using a shotgun approach⁴⁷ and dideoxy sequencing in M13mp19^{48,49}. Areas of ambiguity were determined by sequencing convenient restriction fragments by the Maxam and Gilbert method⁵⁰. The sequence of the remaining 5' non-coding region and confirmation of the fast NGFA sequence awaits the cloning and sequencing of the genomic gene. b, Comparison of the sequences of the four cysteine-rich repeating elements. The cysteines are boxed in and amino-acid residues which are conserved are in a bold type. Dashes, gaps inserted to maximize similarity.

amino acids, on the other hand, has only 3 cysteines and is essentially neutral in charge. It also lacks the consensus sequence of an ATP-binding site, a characteristic of both the tyrosine and serine or threonine kinases³¹. From its nucleotide and amino-acid sequence the fast NGFR appears to be a unique protein. Comparison with the NBR protein data bank as well as the EMBL and Genbank DNA libraries revealed no significant homologies with any other known protein in either nucleotide or amino-acid sequence. This lack of homology, especially with other growth factor receptors or oncogenes, is perhaps not surprising as NGF is a survival and differentiation factor and not a mitogen.

The difference in size between the core protein of the fast NGFR (42,478) and the mature receptor (~83 K) presumably resides in the carbohydrate moieties on one or both of the asparagine residues available for N-linked glycosylation and on one or more of the available O-linked glycosylation sites.

Comparison with other receptors

Although the fast NGFR is a unique protein it has some features in common with other receptors. Repeating elements in the cysteine-rich region in the extracellular domain are also found in other peptide-binding receptors such as the epidermal growth

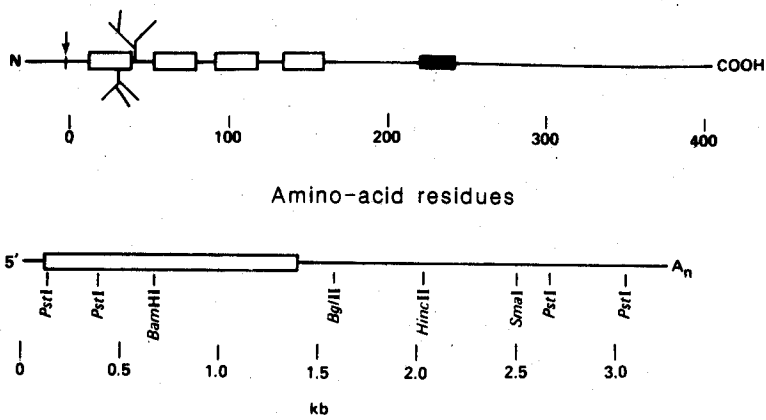


Fig. 6 Restriction map and model of the fast NGFR protein. Bottom, a representation of the transcript and its restriction map. Box, translated portion of the fast NGFR transcript; line, the untranslated portion of the cDNA. Above, a model of the fast NGFR protein. Arrow, site at which the signal peptide is cleaved; black boxes, putative membrane-spanning domain; white box, cysteine repeating elements. The two possible sites for *N*-linked glycosylation are marked by arbitrarily branched side chains.

factor (EGF)³², insulin^{33,34} and low density lipoprotein (LDL)³⁵ receptors. In addition, the EGF, PDGF³⁶ and LDL receptors, and one half of the insulin receptors, like the fast NGFR, have single membrane-spanning domains. The cytoplasmic domain is much shorter than the cytoplasmic domains of the receptors (EGF, PDGF, insulin and *c-fms*³⁷ receptors) with endogenous tyrosine kinase activity. It is more comparable in size to the cytoplasmic domains of the β -adrenergic (C-terminal segment)³⁸ and LDL receptors which also lack this activity. As in the β -adrenergic receptor the cytoplasmic domain of the fast NGFR is rich in serine and threonine residues. Some of these residues in β -adrenergic receptor are phosphorylated by a kinase only when an agonist is bound to the receptor³⁹. Phosphorylation of the human fast NGFR receptor on serine and threonine residues has been reported; however the phosphorylation appears to be insensitive to NGF²¹. The fast NGFR is one of the smaller receptors to be characterized. Thus far only the interleukin-2 (IL-2) receptor, containing 251 amino-acid residues, is smaller^{40,41}. This receptor is similar to the fast NGFR in that approximately half its apparent mass is carbohydrate and it displays two affinities of binding for its ligand IL-2.

The most distinctive feature of the fast NGFR compared to other growth factor receptors is the lack of an ATP binding site in the cytoplasmic domain, suggesting that the binding of NGF to the fast NGFR does not activate an endogenous kinase in

the receptor. The key to the signal transduction mechanism of NGF therefore lies in the difference between the structures of the fast and the slow NGFR. Several models can be proposed to account for this difference: (1) they result from differential splicing of a common precursor mRNA; (2) they arise from separate but homologous genes; (3) they share a common NGF-binding subunit (namely the fast NGFR) or (4) they have no relation other than their ability to bind NGF. The finding that the fast NGFR cDNA hybridizes to a single mRNA species in cells (PC12 and sensory neurons) that express both types of receptor argues against the first two possibilities. It seems likely, therefore that the two receptors share the fast NGFR as a common binding subunit and that the slow NGFR contains a second subunit of $M_r \sim 60K$. The involvement of a *ras*-like gene product in the pathway of action of NGF raises the possibility that the second subunit is one of the family of G proteins.

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Note added in proof: Since submission of this article a very similar sequence has been reported for the human low-affinity NGFR (D. Johnson *et al. Cell* 47, 545-554; 1986).

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