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 described[1] and its physiologically relevant flow from target tissue to neuronal cell body is well characterized[2], 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 body[6]. 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 ~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 slowly[9]. 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-100[9]. Agents which cluster the receptor, such as wheat germ agglutinin[10–12] or antibodies to NGF[12], convert the fast type to a slow type of NGFR, suggesting a relationship between the types of NGFR[13]. The slow and fast NGFR when crosslinked with ^{125}I-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 chain[14]. 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.

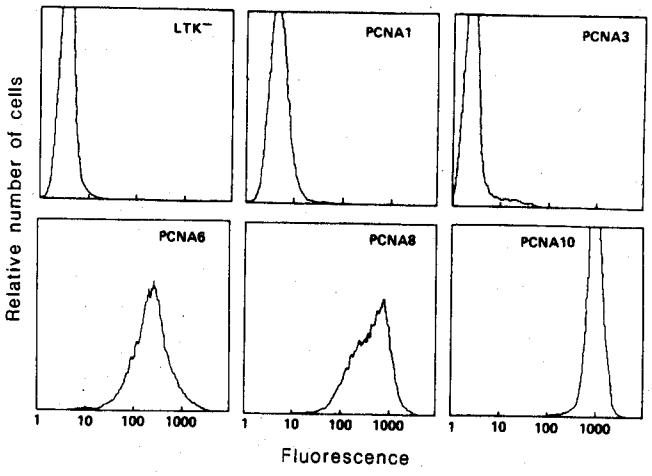


Fig. 1 Amplification of the expression of NGFR in the PCNA cell line. The first panel shows a control staining of LTK$^-$ cells. The remaining panels show the amplification of NGFR expression in the PCNA cells after repeated rounds of sorting. The number attached to the cell line name indicates the number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

Methods. LTK$^-$ cells (10^6) were transfected with 20 μg of PC12 genomic DNA and 1 μg of pCHTK using a modified $CaPO_4$ procedure of Wigler *et al.*[18,19]. Stable transfectants were selected by growth in hypoxanthine, aminopterin and thymidine (HAT) medium[19]. 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 μl of staining medium[19] containing 1 μg of the anti-NGFR monoclonal antibody, MC192 (ref. 20). After 30 min of incubation at 4 °C the cells were washed once with 200 μl of staining medium and resuspended in 50 μl of 4 °C staining medium plus 1 μg 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 μl of staining medium. The stained cells were then resuspended in 200 μl of staining medium, passed through a 50 μm 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 medium[18]. To detect transfectants that expressed the rat fast NGFR the LTK$^+$ cells were stained with MC192, a mouse monoclonal antibody against the fast NGFR[20], and fluorescein-conjugated goat anti-mouse immunoglobulin G

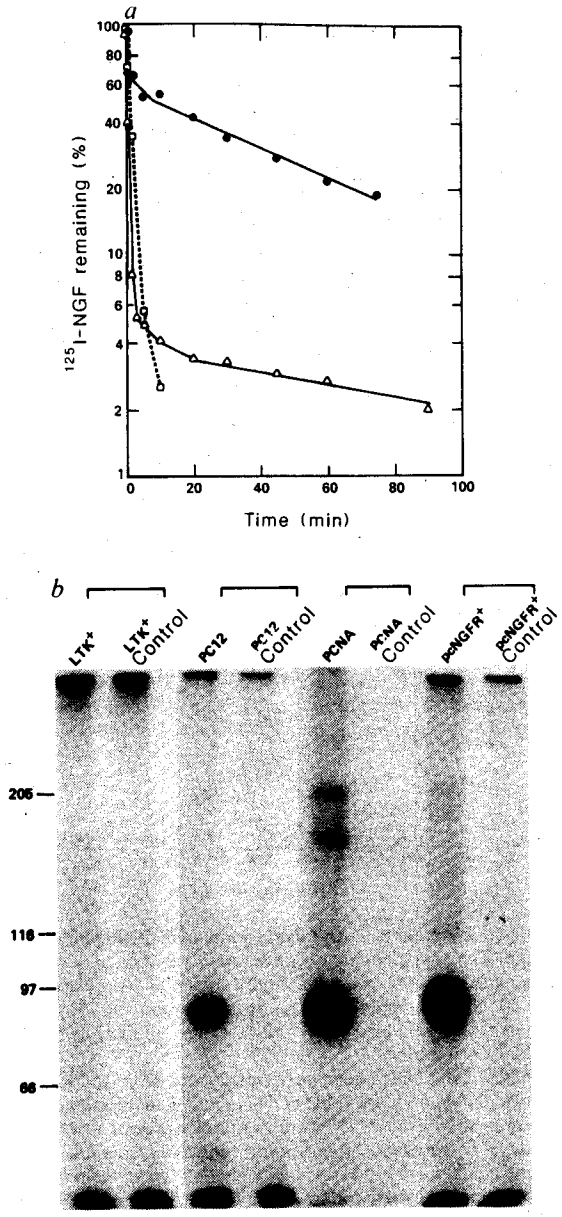


Fig. 2 Characterization of the NGFR in PC12 and transfected cell lines. *a*, Dissociation kinetics of PC12 (●), PCNA (△) and pcNGFR$^+$ (□) cells. Results are expressed as the percentage of the initial specific binding before dissociation. PCNA and pcNGFR$^+$ cells were washed once, harvested in Ca^{2+} Mg^{2+}-free PBS, 1 mM EDTA, *p*H 7.4, and washed in 10 mM HEPES/Krebs-Ringer saline, *p*H 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 (2×10^6 ml^{-1}), PCNA cells (0.5×10^6 ml^{-1}), and pcNGFR$^+$ cells (2×10^6 ml^{-1}) were incubated with 100 pM ^{125}I-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 assayed[9]. *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. LTR$^+$ cells (12×10^6), PC12 cells (14×10^6), PCNA cells (13×10^6) and pcNGFR$^+$ cells (11×10^6) were surface radioiodinated[42] 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 10,000*g* 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 ^{125}I-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 (2.3×10^6 per cell) more than 10-fold greater than on PC12 cells. Analysis of the immunoprecipitates of ^{125}I-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 ^{32}P-labelled PCNA cDNA (made, in turn, from PCNA poly(A)$^+$ RNA) with a 20-fold excess of LTK$^+$ poly(A)$^+$ RNA[24] to remove constitutive LTK$^+$ cDNA. The remaining ^{32}P-labelled cDNA (10% of total cDNA) was used to screen 60,000 colonies of a PCNA cDNA library in pUC9[25–27]. 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

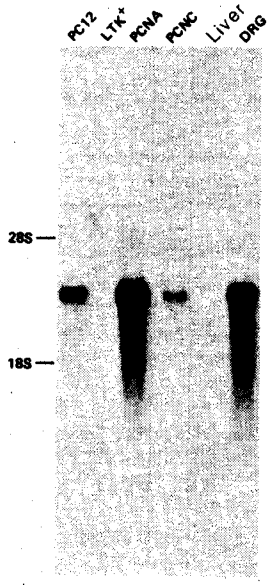


Fig. 3 Northern analysis of the NGFR cDNA clone. Total RNA[44] (10 μg) from PC12 cells, LTK$^+$ cells, PCNA cells, PCNC cells, rat liver and newborn rat dorsal root ganglia (DRG) were each separated on a 0.8% formaldehyde agarose gel[45] and blotted to a nylon membrane according to the manufacturer's instructions (Hybond, Amersham Corp.) A ^{32}P-labelled cDNA probe (~30×10^6 c.p.m.) was made from the putative NGFR cDNA clone insert (pNGFR.1), using random primers[46] and hybridized with the blot in 10 ml of 50% formamide, 5× SSPE, 5× 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×SSC and 0.1% SDS at 65 °C and subjected to autoradiography[25].

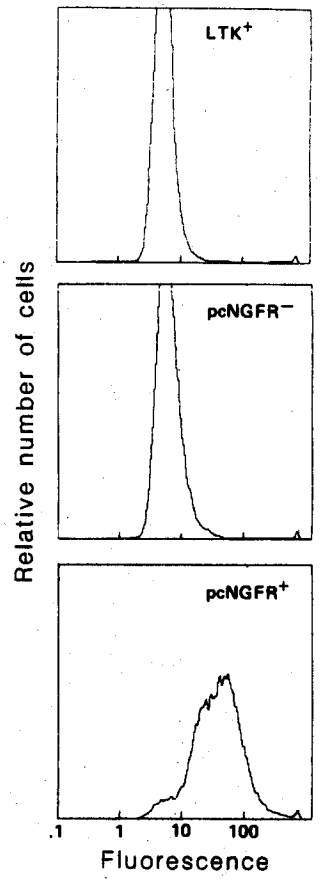


Fig. 4 Transfection of the full length NGFR cDNA in an expression vector into mouse L cells. The complete insert from the putative NGFR cDNA clone, pNGFR.1, was excised with *Eco*RI and *Hin*dIII and blunt-ended with the large fragment of *Escherichia coli* DNA Pol I[25]. The insert was then cloned in 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 μg), pcNGFR$^+$ and pcNGFR$^-$, were independently transfected with 1 μg of pCHTK and 20 μg 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 IgM[19]. The stained cells were then analysed using the FACS.

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.

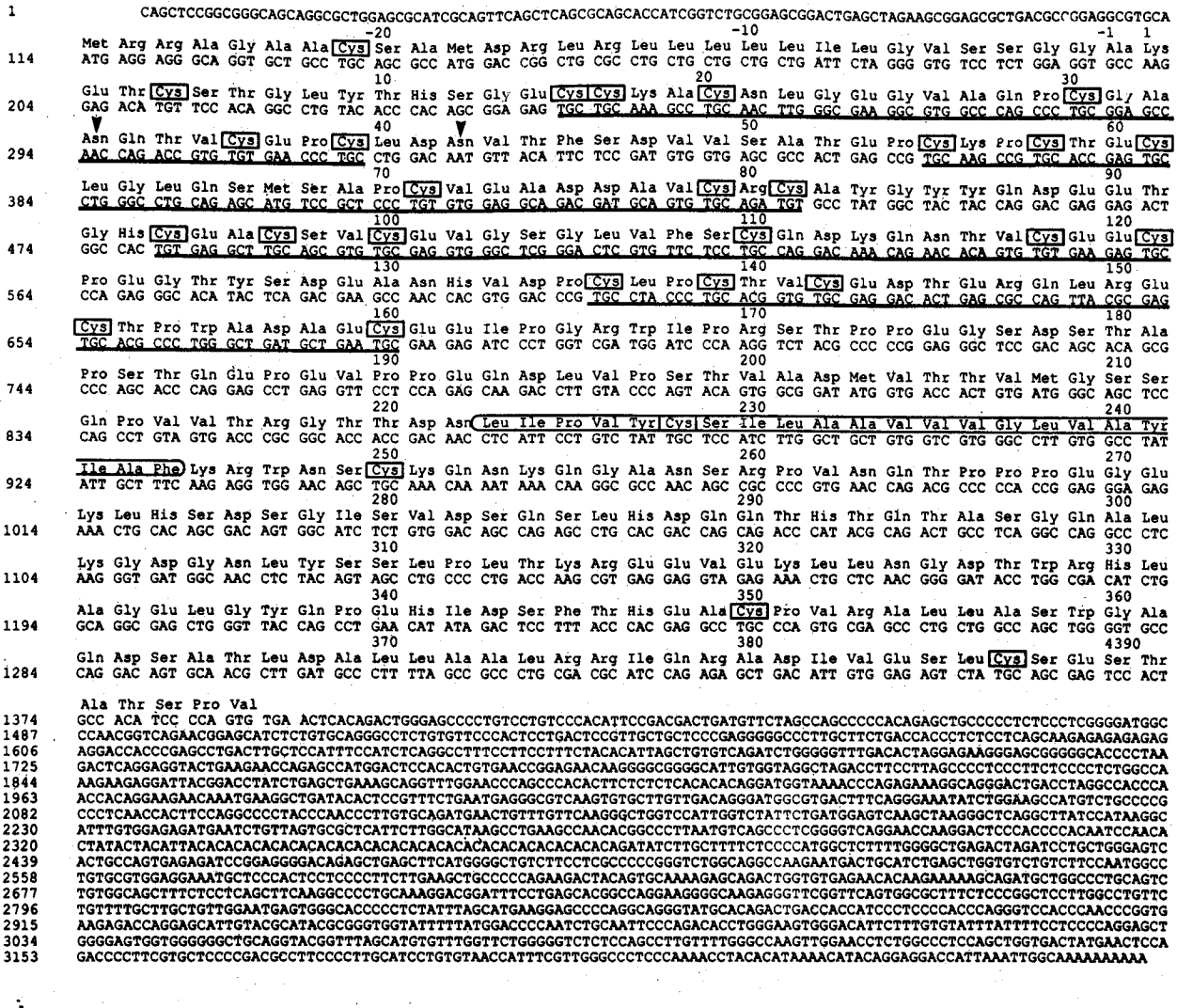
The 3.4-kb clone was inserted into the simian virus 40(SV40)-based vector, pcDL1, in sense and antisense directions and the two subclones (pcNGFR$^+$ and pcNGFR$^-$) independently transfected into LTK$^-$ cells. The FACS analysis of both LTR$^+$ cells and the transfectant with pcNGFR$^-$ after HAT selection 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 ~17,000 receptors per cell. The rate of ^{125}I-NGF dissociation was also characteristic of the PC12 fast NGFR (Fig. 2*a*) and immunoprecipitation of ^{125}-I-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. 2*b*). 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. 5*a*. A model of the fast NGFR protein and a restriction map of the pNGFR.1 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 sequence[28]. Cleavage of this signal peptide results in a mature peptide containing 396 amino-acid residues with M_r 42,478.

Analysis of the hydropathy of the predicted fast NGFR peptide[29] 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 glycosylation[30] and is very acidic. It contains four repeating elements in which the positions of the cysteine residues are highly conserved (Fig. 5*b*). The intracellular domain of 151

a



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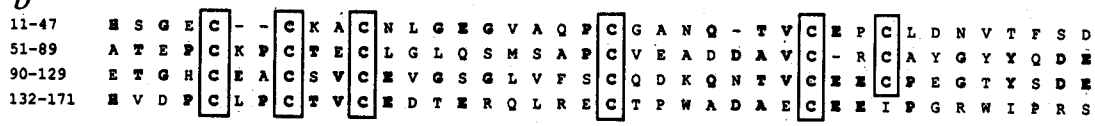

Fig. 5 *a*, Nucleic acid sequence of pNGFR.1 cDNA and the predicted amino-acid sequence of the rat fast NGFR. The nucleotides are numbered on the left and the amino-acid residues are numbered above the sequence. Arrows, two possible sites of asparagine-linked glycosylation; round-ended box, putative membrane-spanning domain. The cysteine residues are in rectangles and the four repreating elements are underlined. The sequence was obtained on both strands primarily using a shotgun approach[47] and dideoxy sequencing in M13mp19[48,49]. Areas of ambiguity were determined by sequencing convenient restriction fragments by the Maxam and Gilbert method[50]. 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 repeatng 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 kinases[31]. 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

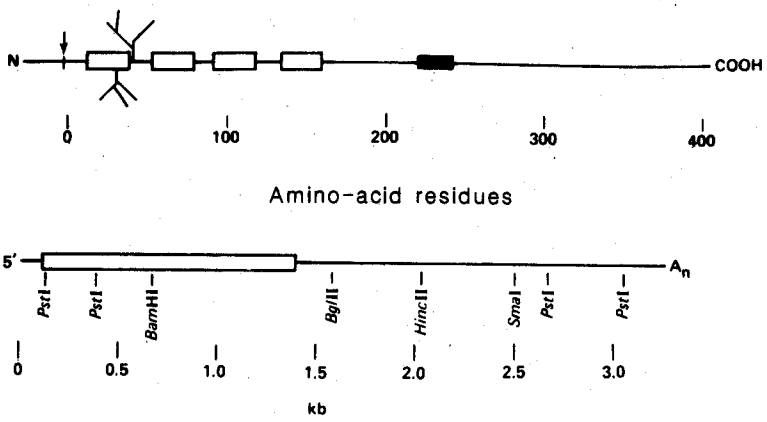

Fig. 6 Restriction map and model of the fast NGFR protein. Bottom, a representation of the transcript and its restriction map. Box, translated portion of the fast NGFR transcript; line, the untranslated portion of the cDNA. Above, a model of the fast NGFR protein. Arrow, site at which the signal peptide 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, PDGF[36] 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 receptor[39]. Phosphorylation of the human fast NGFR receptor on serine and threonine residues has been reported; however the phosphorylation appears to be insensitive to NGF[21]. 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 $M_r \sim 60$K. 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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