Expression of Functional Nerve Growth Factor Receptors After Gene Transfer

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Nerve growth factor (NGF) interacts with both high affinity (K_d = 10^{-10}–10^{-11} *M*) and low affinity (K_d = 10^{-8}–10^{-9} *M*) receptors; the binding of NGF to the high affinity receptor is correlated with biological actions of NGF. To determine whether a single NGF binding protein is common to both forms of the receptor, a full-length receptor cDNA was introduced in the NR18 cell line, an NGF receptor-deficient variant of the PC12 pheochromocytoma cell line. The transformant displayed (i) both high and low affinity receptors detectable by receptor binding; (ii) an affinity cross-linking pattern with ^{125}I-labeled NGF similar to that of the parent PC12 cell line; and (iii) biological responsiveness to NGF as assayed by induction of c-*fos* transcription. These findings support the hypothesis that a single binding protein is common to both forms of the NGF receptor and suggest that an additional protein is required to produce the high affinity form of the NGF receptor.

THE MANY PHYSIOLOGICAL EFFECTS of NGF on the survival and maintenance of selective neuronal populations (*1*) are directly mediated by binding of NGF to a specific cell surface receptor. The NGF receptor is represented by two kinetic classes that differ by 100-fold in the rate of dissociation of NGF (*2, 3*). The biochemical basis of these forms is not understood.

Molecular cloning of the human NGF receptor indicates the receptor gene is a single copy gene (*4*) on chromosome 17 (*5*) and contains six exons spanning over 23 kb (*6*). A single receptor mRNA of 3.8 kb has been detected in responsive sympathetic and sensory neurons and cholinergic cells in the basal forebrain (*7*), as well as in some neuroblastoma and melanoma cells (*4*).

Introduction of cDNA clones encoding the NGF receptor into fibroblasts and melanoma cells generates cells that express only the low affinity form of the receptor (*4, 8, 9*). As many as 500,000 receptors per cell can be observed after transfection; however, none of the lines display any detectable responses after NGF treatment. The lack of responses to NGF in cells expressing abundant NGF receptors suggests that either these cells do not possess the proper cellular environment to respond to NGF or the cDNA clone for the NGF receptor represents only the low affinity form of the receptor.

To distinguish between these possibilities, a full-length human receptor cDNA was expressed in cells more likely to be responsive to NGF. NR18 cells were used because (i) they are a mutant cell line derived from the NGF-responsive rat pheochromocytoma line, PC12 (*10*), and (ii) they lack receptors for NGF and do not respond to NGF (*11*). A recombinant retrovirus containing a full-length cDNA for the NGF receptor (E1) was produced by an amphotrophic packaging line, and this retrovirus was used to infect NR18 cells (*12*). A stable transformant expressing the human receptor was identified after neomycin selection and purified by rosetting with monoclonal antibodies against the human NGF receptor (*13*).

Southern and Northern blot analysis of the transformant line, NR1-1, indicated that this line carried an integrated copy of the human receptor cDNA and produced a transcript of 4.1 kb, consistent with the longer size of the mRNA produced from the retroviral long terminal repeat (LTR) sequence (Fig. 1). No detectable rat receptor mRNA was observed in parental NR18 cell line. Activation of the endogenous rat receptor by retroviral insertion has never been observed.

The lack of endogenous NGF receptors in NR18 cells was confirmed by affinity cross-linking of ^{125}I-labeled NGF to intact cells. Whereas cross-linking with ethyldimethylaminopropyl carbodimide (EDAC) produced a major species of 90 to 100 kD in PC12 cells and in the human melanoma cell line, Hs294 (*14*), no cross-linking was observed in the NR18 cell (Fig. 2A). Immunoprecipitation of cross-linked receptors with monoclonal antibodies specific for the human (*13*) and rat (*15*) receptors demonstrated that the transformed NR1-1 cells exhibited only human receptors (Fig. 2B). The specificity of the antibodies can be observed in receptors immunoprecipitated from rat PC12 cells versus the human melanoma Hs294 cell line.

To determine whether the introduced human receptor can form high affinity binding sites, Scatchard plot analysis was carried out by means of a membrane binding assay (Fig. 3). Crude membranes were prepared from cells and assayed by binding to ^{125}I-labeled NGF (*16*). PC12 membranes displayed high affinity receptors (K_d = 8×10^{-11} *M*; 8,000 sites per cell), and low affinity receptors (K_d = 5×10^{-8} *M*; 150,000 sites per cell). In contrast, a fibroblast cell line (2_1) generated by transfection of E1 cDNA into mouse L cells (*8*) demonstrated a single, low affinity binding site (K_d = 4×10^{-9} *M*; 45,000 sites per cell). Retroviral infection of fibroblasts likewise led to a single class of low affinity receptors (*17*). In agreement with the previous results, specific binding was not observed with membranes prepared from the NR18 cell line. However, the membranes isolated from the NR1-1 line contain both high (K_d of 6×10^{-11} *M* and 1,800 sites per cell) and low affinity receptors (K_d of 2×10^{-8} *M* and 60,000 sites per cell) with similar affinities (*18*) to the receptor class found in normal PC12 membranes (Fig. 3).

The ability of the human NGF receptor to confer biological responsiveness in the NR1-1 cells was examined. Treatment of NR1-1 cells with physiological concentrations of NGF did not give any of the delayed morphological or differentiative responses resembling the responses of PC12 cells to NGF. However, one of the early responses to NGF, induction of protooncogene c-*fos* (*19*), was observed. NR1-1 cells treated with NGF (50 ng/ml) for 30 min responded by inducing c-*fos* mRNA synthesis (Fig. 4A).

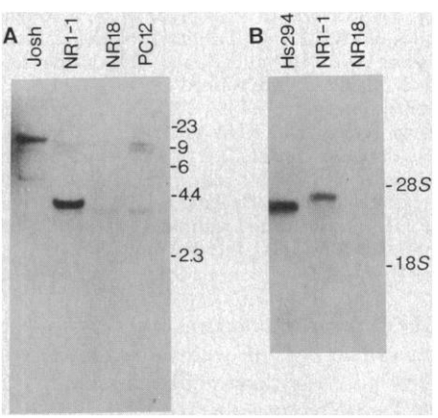

Fig. 1. Introduction and expression of the human NGF receptor cDNA in NR18 cells after retroviral infection. (**A**) High molecular weight DNA was isolated from a human cell line (Josh), as well as from NR1-1, NR18, and PC12 cells, digested with Eco RI, separated by electrophoresis, transferred to nitrocellulose, and hybridized to nick-translated E1 receptor cDNA sequences (*8*). The faint bands in the PC12 and NR18 DNA represent the rat receptor sequences. (**B**) RNA analysis of the human melanoma cell line, Hs294, NR1-1, and NR18 cell probed with nick-translated human El receptor cDNA.

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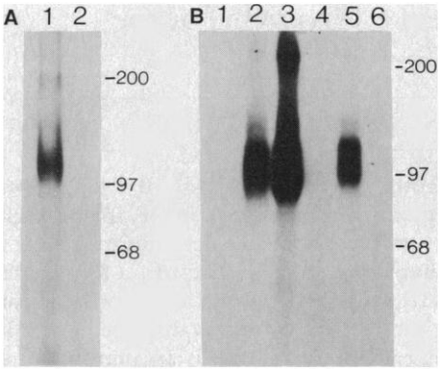

Fig. 2. Affinity cross-linking and immunoprecipitation of NGF receptors in cultured cells. (**A**) NGF receptors were labeled by cross-linking to ^{125}I-labeled NGF (1 n*M*) with 4 m*M* EDAC, then extensively washed. After addition of SDS buffer (0.1*M* tris, *p*H 6.8, 10% glycerol, 1% SDS, 0.001% bromophenol blue, 10% 2-mercaptoethanol), the samples were subjected to electrophoresis on an 8% polyacrylamide-SDS gel. Lane 1, PC12 cells; lane 2, NR18 cells. (**B**) Immunoprecipitation was carried out by incubating the cross-linked cells, which had been extracted with 2% octylglucoside and phosphatidylcholine, with 15 μl of mouse ascites fluid. The mice contained intraperitoneal tumors derived from the hybridomas ME20.4 (from the ATCC), specific for the human receptor (*13*), or 192-IgG, specific for the rat receptor (*15*). After the addition of 30 μg of rabbit antibody to mouse IgG and protein A–Sepharose, the bound complexes were washed extensively in phosphate-buffered saline, extracted with SDS buffer, and subjected to electrophoresis. Protease inhibitors were present throughout the procedure. Lanes 1 and 2, PC12 cell extract immunoprecipitated with ME20.4 and 192-IgG, respectively. Lanes 3 and 4, Hs294 cell extract immunoprecipitated with ME20.4 and 192-IgG, respectively. Lanes 5 and 6, NR1-1 cells immunoprecipitated with ME20.4 and 192-IgG, respectively.

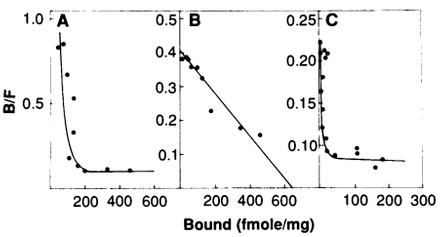

Fig. 3. Equilibrium binding analysis of NGF receptors in cell membrane preparations. Binding of ^{125}I-labeled NGF was assessed by filter binding to crude membrane preparations (*16*). Each binding reaction was carried out in triplicate in the presence and absence of an excess of unlabeled NGF. Only binding values above 50% specific binding were used in the analysis. The LIGAND program was utilized to analyze the data. B/F, bound/free. Membranes were analyzed from (**A**) PC12 cells; (**B**) 2_1 fibroblast cells; (**C**) NR1-1 cells. Panel (C) is a composite Scatchard of two separate binding experiments.

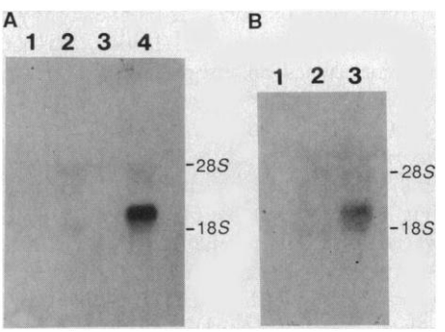

Fig. 4. NGF receptor mediates the induction of c-*fos* mRNA by NGF. Confluent cells were treated with NGF (50 ng/ml) for 30 min at 37°C. Total RNA was isolated and separated by electrophoresis on a 1% agarose gel containing 2.2*M* formaldehyde. Expression of c-*fos* mRNA was assayed by hybridization to v-*fos* probe at 50% formamide, 5× SSCPE, 1× Denhart's solution, salmon sperm DNA (100 μg/ml), and 10% dextran sulfate. The filters were washed to 1× SSC at 55°C and autoradiographed at −70°C with an intensifying screen. (**A**) RNA isolated from lane 1, NR18 cells; lane 2, NR18 cells treated with NGF; lane 3, NR1-1 cells; lane 4, NR1-1 cells treated with NGF. (**B**) Lane 1, NR1-1 cells; lane 2, NR1-1 cells treated with both NGF (50 ng/ml) and monoclonal antibody ME20.4; lane 3, NR1-1 cells treated with NGF (50 ng/ml).

The c-*fos* transcription was dependent on the interaction of NGF with the human receptor, since monoclonal antibodies specific for the human receptor that block NGF binding (*13*) could eliminate the induction of c-*fos* expression (Fig. 4B). The c-*fos* response in the NR1-1 line, coupled with previous observations that the increased rate of transcription of c-*fos* is mediated by NGF binding to the high affinity receptor (*20*), strongly argues that the cloned human receptor cDNA can give rise to functional NGF receptors in appropriate cells.

Therefore, we conclude that the NGF receptor gene gives rise to a single mRNA that represents both the high and low affinity receptors. No evidence has been found for alternative NGF receptor genes or differential mRNA processing (*21*). A single receptor mRNA species has been detected in neurons and in cultured cells expressing both high and low affinity receptors (*4, 7–9*). We have shown that a full-length receptor cDNA can generate both high and low affinity NGF receptors. These results are consistent with previous cross-linking studies of the NGF receptor in PC12 cells, suggesting that both kinetic classes are represented by the same protein (*22*).

These experiments also imply that the wide variety of responses to NGF, including changes in gene expression, synthesis of enzymes in the neurotransmitter pathway, and electrical properties, are the result of a multistep cascade. It is likely that NR18 cells contain defects that preclude the recapitulation of many of NGF's responses.

The ability to generate high affinity NGF receptors in certain populations of cells is reminiscent of the behavior of the interleukin-2 (IL-2) receptor, which displays only high affinity IL-2 binding in appropriate T lymphocytes (*23*). In that system, an accessory molecule is required for the high affinity IL-2 receptor (*24*). The expression of high affinity NGF receptors only in selected cells that are derived from the neural crest suggests that a separate protein present only in certain populations of cells may be required for the formation of the high affinity state. This hypothesis is supported by the observation of higher molecular weight complexes representing the NGF receptor; these have been found by chemical cross-linking with lipid-soluble reagents and in partial purification studies (*22, 25*). Although posttranslational modifications may account for a difference of affinity, there is presently no indication that a posttranslational event influences receptor affinity for NGF. The NR18 cells now provide an opportunity to investigate further the molecular basis of the high affinity NGF receptor.

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12. Helper-free virus stocks containing a full-length NGF receptor sequence were generated from a packaging cell line (PA317) transfected with pMVE1 (*8*). A stably transfected cell line (PAE1c) was isolated after neomycin selection and rosette analysis (*4*). Culture medium from PAE1c cells was used to infect NR18 cells in the presence of polybrene (8 μg/ml).
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16. Crude membranes were isolated after Polytron homogenization of cultured cells suspended in 1 m*M* tris, *p*H 8, 1 m*M* EDTA. Nuclei were removed after centrifugation at 3,000 rev/min for 5 min and

membranes were pelleted after a 30-min centrifugation at 45,000 rev/min. The membranes were resuspended in the same buffer at a concentration of 5 to 10 mg/ml and frozen at −70°C or used immediately. NGF was iodinated by lactoperioxidase treatment to 2800 to 3800 cpm/fmol (*22*). Equilibrium binding to ^{125}I-labeled NGF was carried out in a 100-μl reaction containing 10 to 40 μg of membranes in 50 m*M* Hepes, *p*H 8, BSA (1 mg/ml Sigma) for 60 min at 30°C. Binding was concluded with 1.5 ml of wash buffer [20 m*M* $NaHPO_4$, *p*H 7.4, 50 m*M* NaCl, BSA (1 mg/ml), protamine sulfate (1 mg/ml), 37°C] and each reaction was filtered under vacuum through Millipore HVLP filters (0.45 μm). After three 10-ml rinses in wash buffer, the filters were counted. Nonspecific binding was assessed by including unlabeled NGF to a final concentration of 800 n*M*. Specific binding ranged from 60 to 90% of total binding.

17. Psi2 fibroblast cells were infected with a recombinant retrovirus from PAE1c (*12*) and a neo$^+$, NGF receptor-expressing clone was isolated. Equilibrium binding of ^{125}I-labeled NGF to membranes isolated from this cell line indicated that only low affinity receptors were present.
18. The minor differences noted in K_d of the low affinity sites measured on intact cells ($K_d = 1 \times 10^{-9} M$) and cell membranes ($K_d = 2$ to $5 \times 10^{-8} M$) may be related to a loss of cytoskeletal association in membrane preparations, as described by S. Buxser *et al.*, *J. Biol. Chem.* **260**, 1917 (1985).
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26. We thank M. Bothwell for the NR18 cell line, T. Curran for the v-*fos* probe, E. Johnson for the 192-IgG hybridoma, and P. Gupta for assistance. Supported by the NIH (NS-21072 and HD23315). B.L.H. was a recipient of the Rose Kloss Fellowship and L.S.S. received a Physician-Scientist Award from the American Heart Association. L.S.S. and M.V.C. were Cornell Scholars in Biomedical Science and M.V.C. was supported by the Hirschl/Caulier Trust and the Dorothy Rodbell Cohen Foundation.

25 August 1988; accepted 11 November 1988

Increased Expression of DNA Cointroduced with Nuclear Protein in Adult Rat Liver

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DNA and nuclear proteins were transferred into cells simultaneously at more than 95% efficiency by means of vesicle complexes. The DNA was rapidly transported into the nuclei of cultured cells, and its expression reached a maximum within 6 to 8 hours after its introduction. Moreover, when the plasmid DNA and nuclear protein were cointroduced into nondividing cells in rat liver by injection into the portal veins of adult rats, the plasmid DNA was carried into liver cell nuclei efficiently by nuclear protein. The expression of the DNA in adult rat liver, on introduction of the DNA with nuclear protein, was more than five times as great as with nonnuclear protein.

INTRODUCTION OF FOREIGN GENES into cultured cells by methods such as calcium-phosphate precipitation, electroporation, virus vector, and liposome fusion has provided new insights into the functions of many types of cells and macromolecules (*1*). These methods introduce genes into the cell cytoplasm; however, the genes cannot be expressed unless the DNA reaches the nucleus.

Recently, the migration of nuclear proteins into the nucleus has been investigated (*2, 3*), but the mechanism of transport of exogenous DNA into the nucleus remains to be elucidated (*4*). Sendai virus (HVJ) was used to fuse DNA-loaded ganglioside liposomes with red blood cell (RBC) membrane vesicles that contain proteins, so that DNA and proteins can be simultaneously introduced into the same cells efficiently. The procedure for constructing the vesicle complexes is shown in Fig. 1. HVJ-liposomes (*5*) containing a thymidine kinase (TK) gene were mixed with RBC membrane (*5, 6*) containing the fluorescein protein phycoerythrin (*7*) to form vesicle complexes. When these complexes were added to cultured mouse Ltk$^-$ cells, more than 95% of the cells showed red fluorescence in their cytoplasm and incorporated [^{3}H]thymidine into their nuclei (*8*).

This delivery system was then used to cointroduce pBR-SV40 DNA with either nonhistone chromosomal protein [high-mobility group–1 (HMG-1)] or bovine serum albumin (BSA) into Ltk$^-$ cells. Within 6 hours of treatment of cells with the vesicle

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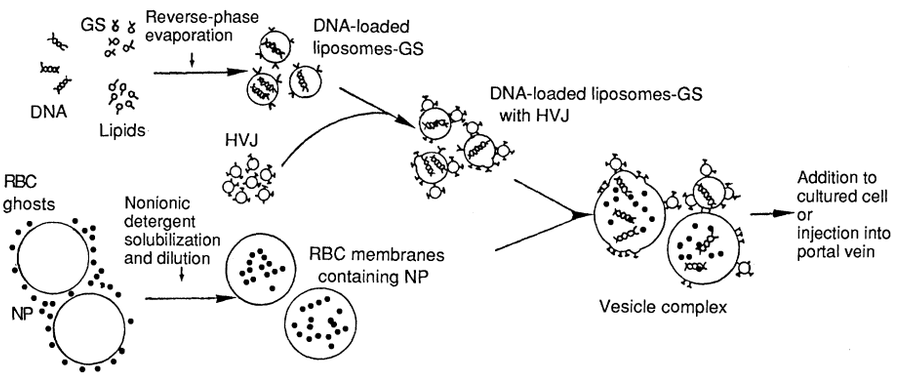

Fig. 1. The procedure for simultaneous introduction of plasmid DNA and nuclear proteins into cultured cells or adult rat liver. GS, gangliosides; NP, nuclear protein. The first step was the interaction of DNA-loaded liposomes with HVJ. Liposomes containing DNA and gangliosides were prepared by the reverse-phase evaporation method (*5, 17*), which traps about 30% of the plasmid DNA in liposomes (*5*). Proteins cannot be incorporated because of the use of organic solvents. About 50 μg of plasmid DNA (about 20 μg in the case of pTK4) was entrapped in 2 ml of liposome suspension (10 mg of lipids). Liposomes suspended in 2 ml of balanced salt solution were mixed with 2 ml of HVJ (about 15,000 HAU hemagglutination units per 0.5 ml) and 1 m*M* $CaCl_2$ and incubated at 4°C for 20 min and then at 37°C for 40 min. Next, 600 μl of packed volume of human RBC membranes (RBC ghosts) prepared as described (*5, 6*) was mixed with 300 μl of HMG-1 (10 mg/ml), BSA (Sigma) (30 mg/ml), rabbit IgG (CPL) (60 mg/ml), or IgG-Tpep (*14*) (8 mg/ml). Then RBC membrane vesicles containing proteins were prepared by the detergent solubilization and dilution method (*5, 6*). These vesicles (about 320 μl) were incubated with 4 ml of HVJ-liposomes at 4°C for 20 min and then at 37°C for 3.5 hours with shaking. Cultured cells (10^6 to 10^7 in suspension and 10^5 in monolayer) were incubated with a 2-ml suspension of the vesicle complexes (about 4×10^8) at 4°C for 10 min and then at 37°C for 60 to 120 min. For in vivo introduction of genes, 3 ml of the vesicle complexes (about 6×10^8) was injected into the portal vein of each Sprague-Dawley rat (6 to 8 weeks old).