

Characterization of the Binding Properties and Retrograde Axonal Transport of a Monoclonal Antibody Directed Against the Rat Nerve Growth Factor Receptor

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ABSTRACT We have demonstrated *in vitro* and *in vivo* the specific binding of a monoclonal antibody to the rat nerve growth factor (NGF) receptor. Previous work had shown that this antibody, designated 192-IgG, does not compete with NGF for binding to the NGF receptor of PC12 cells, but instead interacts with the receptor to increase NGF binding to PC12 cells (Chandler, C. E., L. M. Parsons, M. Hosang, and E. M. Shooter, 1984, *J. Biol. Chem.*, 259:6882–6889). In the present study, a solid-phase separation assay verified the specific formation of a ternary complex of 192-IgG, the NGF receptor, and NGF: ¹²⁵I-labeled 192-IgG precipitated from solution only when incubated with both solubilized NGF receptor and NGF covalently linked to a solid phase (Sephacrose 4B). Filtration assays using plasma membrane preparations of various tissues showed strict correlation of ¹²⁵I-192-IgG and ¹²⁵I-labeled NGF binding; only membranes obtained from superior cervical ganglion bound significant amounts of the monoclonal antibody and NGF. Injection of ¹²⁵I-192-IgG into the rat anterior eye chamber led to accumulation of intact antibody molecules in the ipsilateral superior cervical ganglion, indicating retrograde axonal transport of ¹²⁵I-192-IgG from the neuronal termini, located at the iris, to the cell bodies situated in the ganglion. The time course and saturation characteristics of ¹²⁵I-192-IgG retrograde transport were very similar to those previously reported for ¹²⁵I-NGF transport, indicating that 192-IgG can be internalized and transported by the same mechanisms as is NGF. Consistent with results of the *in vitro* binding assays, 192-IgG and NGF failed to compete for retrograde transport and were actually co-transported. Retrograde axonal transport of 192-IgG appears to be species specific, since ¹²⁵I-192-IgG was transported in the rat, but not in mice, gerbils, hamsters, or guinea pigs. These results establish monoclonal antibody 192-IgG as a specific probe for the rat NGF receptor *in vitro* and *in vivo*.

Nerve growth factor (NGF)¹ is a polypeptide neurotrophic agent that is essential for survival and maintenance of sympathetic neurons and some sensory neurons, both *in vivo* and in dissociated cell culture (for review see references 1 and 2). Organs innervated by sympathetic nerves produce minute quantities of NGF (3), which binds to specific receptors on the neurons' terminal fibers. NGF is then internalized and retrogradely transported within the axons to the cell bodies (4–6). The biochemical events through which NGF promotes neuronal survival and function are, as yet, unknown.

¹ *Abbreviations used in this paper:* EGF, epidermal growth factor; NGF, nerve growth factor; SCG, superior cervical ganglion (ganglia).

The NGF receptor must play at least one critical role in the biology of NGF, namely, binding the factor to the proper neuronal termini and thereby targeting it to the appropriate cells. By analogy to the functions described for the insulin receptor (7) and the epidermal growth factor (EGF) receptor (8), the NGF receptor may be directly involved in mediating at least some of the biological effects of NGF. Specific NGF receptors are found in superior cervical ganglion (SCG) neurons (9), dorsal root ganglion neurons (10), and some other cells of neural crest origin, in particular melanomas (11) and the PC12 cell line from a rat pheochromocytoma (12). Many binding data have been accumulated, and there is a report of the purification of the receptor from a human melanoma

(13); there is, nevertheless, little information regarding the *in vivo* actions of the NGF receptor. A tool useful in obtaining such information would be a ligand that binds specifically to the NGF receptor, but which, unlike NGF itself, is not normally required by the cells for survival.

A monoclonal antibody, designated 192-IgG, was developed as a specific probe for the NGF receptor (14). The hybridoma clone was generated by the fusion of Sp2/O-Ag14 myeloma cells with BALB/c splenocytes from mice immunized with detergent-solubilized plasma membrane proteins of PC12 cells. 192-IgG does not bind NGF, nor does it compete with NGF for binding to PC12 cells. Consistent with 192-IgG specifically binding to only the NGF receptor, the antibody binds to the same number of sites on PC12 cells as does NGF, and it increases the apparent affinity of NGF binding by 2.5- to 4-fold. Cross-linking of ¹²⁵I-labeled 192-IgG to PC12 cells using hydroxysuccinimidyl-4-azidobenzoate and subsequent polyacrylamide electrophoresis and autoradiography led to the appearance of a major band of molecular weight 160,000; this molecular weight is consistent with the cross-linking of one heavy and one light chain of 192-IgG (a total M_r of 75,000) to the NGF receptor ($M_r = 74,000$). Antigen detection using a protein transfer technique (15) failed to produce further evidence that the 192-IgG binds the NGF receptor itself and not a separate cell surface protein which is present in equal numbers to the NGF receptor and which interacts with the receptor protein. These results strongly suggested, but did not prove, that the antibody binds to the NGF receptor molecule; the 192-IgG was therefore termed an "NGF receptor interactive monoclonal antibody."

We have performed experiments to demonstrate the specific binding of monoclonal antibody 192-IgG to NGF receptors *in vitro* and *in vivo*. A solid-phase separation assay using solubilized plasma membrane proteins of rat SCG indicated the simultaneous binding of 192-IgG and NGF to the NGF receptor. Binding of the monoclonal antibody to plasma membrane preparations from various tissues showed strict correlation with NGF binding. Retrograde transport of intracellularly injected ¹²⁵I-NGF is a well-documented process that involves the binding of the protein to its receptor on the nerve termini, internalization into the neuron, and active vectorial translocation to the cell body (2, 4, 16). Our experiments revealed retrograde transport of ¹²⁵I-192-IgG with characteristics similar to that of ¹²⁵I-NGF. We conclude that the 192-IgG is a specific probe for the NGF receptor both *in vitro* and *in vivo*. Some of the experiments reported here have appeared in an abstract (17).

MATERIALS

The 2.5S subunit of NGF was used in all experiments; it was purified from adult male mouse submaxillary glands according to the method of Bocchini and Angeletti (18). The monoclonal antibody, 192-IgG, was purified from ascites by ammonium sulfate precipitation (50%) followed by anion exchange chromatography on DEAE Sephacel (Pharmacia Fine Chemicals, Piscataway, NJ) (19) and gel filtration on a Sephacryl S-200 column (1.5 cm I.D. × 116 cm) equilibrated with PBS (20 mM K₂HPO₄, 160 mM NaCl, pH 7.4). The A chain protein subunit of ricin toxin was purified from castor beans (Hummert Seed Co., St. Louis, MO) as described previously (20, 21); NGF, 192-IgG, and ricin A chain were >95% pure, as assessed by SDS PAGE (22). Electrophoresis reagents were obtained from Bio-Rad Laboratories, Richmond, CA. All other chemicals, unless noted, were purchased from Sigma Chemical Co., St. Louis, MO.

Iodination Procedure: Both NGF and 192-IgG were iodinated by the lactoperoxidase method (23), as previously described (24). 10 μg of NGF or 100 μg of 192-IgG were each reacted with 1 mCi of Na¹²⁵I (Amersham

Corp., Arlington Heights, IL). The amount of incorporation of I-125 into the proteins ranged from 70 to 90%, corresponding to specific activities of 1.8–2.3 Ci/μmol for NGF and 1.1–1.4 Ci/μmol for 192-IgG. For use in binding assays the labeled ligands were separated from non-incorporated I-125 by chromatography on Sephadex G-25 M (PD-10 columns; Pharmacia Fine Chemicals). The ¹²⁵I-NGF was further processed by filtration through an Amicon Centrilo 50A membrane (pre-soaked in PBS/0.1% Triton X-100) to remove aggregated molecules.

Plasma Membrane Preparation: The SCG were dissected from male Sprague-Dawley rats weighing 150–175 g (Chappel Breeders, St. Louis, MO) and kept frozen at –70°C until use. A plasma membrane-enriched microsomal fraction was prepared from 500–700 ganglia by the method of Costrini and Bradshaw (25). Tissues designated as controls were processed in the same manner. For use in the filtration binding assay the microsomal fraction was suspended in phosphate-buffered saline (PBS). Solubilized NGF receptor (used in the solid-phase separation assay) was obtained by adding to the suspended microsomal fraction sufficient *n*-octyl glucopyranoside (1-*O*-*n*-octyl-β-D-glucopyranoside; Sigma Chemical Co.) to achieve a 2% final concentration, mixing for 1 h at room temperature, then centrifuging for 1 hour at 100,000 *g* to remove insoluble material. Protein concentrations were determined by the Lowry method (26).

Coupling of NGF or Ricin A Chain to Sepharose 4B: Sepharose 4B was activated by reacting it with cyanogen bromide (27): 10 ml of the activated gel was combined with 8.1 mg of NGF in 250 mM sodium bicarbonate/500 mM NaCl, pH 8.5 (coupling buffer). A 5-ml volume of gel was linked with 10 mg of ricin A chain in coupling buffer. The mixtures were turned end-over-end for 6 h at room temperature, then overnight at 4°C. The solid phase of each mixture was pelleted by a 15-min centrifugation at 1,000 *g*, and the liquid was decanted. The amount of NGF remaining in solution was determined to be 0.80 mg by measuring the absorbance at 280 nm ($\epsilon = 1.64$); the amount coupled was therefore calculated to be 7.30 mg (90% efficiency). 7 mg of ricin A chain was linked to Sepharose 4B. The NGF-Sepharose 4B and ricin A chain-Sepharose 4B gels were then each treated with 10 ml of 0.2 M glycine in 100 mM sodium borate, pH 8.5, for 2 h. Finally, each gel was washed with two cycles of coupling buffer followed by 100 mM sodium acetate/500 mM NaCl, pH 4.5.

Solid-Phase Separation Assay: Detergent-solubilized membrane proteins from rat superior cervical ganglia (SCG) (10 μg) were mixed with ¹²⁵I-192-IgG (50,000 cpm, ~25 fmol) in 0.5-ml microfuge tubes (Sarstedt, Inc., Princeton, NJ) on ice for 2 hours. Each mixture also contained 1 mg/ml bovine serum albumin (BSA) and 2% *n*-octyl glucopyranoside in a final volume of 50 μl. A 10-μl aliquot of a 40% (vol/vol) slurry of NGF-Sepharose 4B was added and the mixture was allowed to equilibrate for an additional hour on ice; occasional vortexing of the tubes maintained the NGF-Sepharose 4B in suspension. Each tube was then centrifuged in a Beckman microfuge B (Beckman Instruments, Inc., Palo Alto, CA) for 10 s to sediment the NGF-Sepharose 4B. The supernatant was aspirated, and the pellet was washed three times with 150 μl of PBS/2% *n*-octyl glucopyranoside. The radioactivity remaining in the NGF-Sepharose 4B pellet was measured in a Beckman 4000 gamma counter. To demonstrate that SCG membrane proteins are necessary to link the ¹²⁵I-192-IgG to the NGF-Sepharose 4B, the SCG preparation was omitted, or was replaced with *n*-octylglucoside-solubilized membrane proteins from rat liver (100 μg per tube), or rat lung (30 μg). The specificity of ¹²⁵I-192-IgG binding to the SCG protein was tested by including in the mixture an excess of nonlabeled 192-IgG (200 pmol) or a control monoclonal IgG directed against the EGF receptor of PC12 cells (200 pmol). To prove that ¹²⁵I-192-IgG associates with an SCG protein which binds specifically to NGF, the NGF-Sepharose 4B was replaced with an equal volume of ricin A chain-Sepharose 4B, or, an excess of either soluble NGF (1.3 nmol) or cytochrome *c* (1.5 nmol) was added concurrently with the NGF-Sepharose 4B.

Filtration Binding Assay: The binding of ¹²⁵I-NGF and ¹²⁵I-192-IgG to the plasma membrane-enriched microsomal fraction obtained from different tissues was assayed by using a modification of the method described by Banerjee et al. (9). A membrane preparation (25 μg of protein) of rat SCG, spinal cord, adrenal, liver, lung, or skeletal muscle was mixed with ¹²⁵I-NGF (100,000 cpm) or ¹²⁵I-192-IgG (900,000 cpm) in PBS/0.1% BSA in a total volume of 200 μl. After 40 min at 24°C, 3 ml of ice cold PBS/0.1% BSA/0.05% protamine sulfate was added to each tube and the entire contents were filtered through a Millipore HVL membrane by use of a Millipore manifold and a vacuum pump. Each filter was washed twice with 3-ml aliquots of the PBS/0.1% BSA/0.05% protamine sulfate, and then measured for radioactivity. Parallel assays containing excess nonlabeled NGF (900-fold excess) or nonlabeled 192-IgG (120-fold excess) determined the amount of nonspecifically bound ¹²⁵I-NGF or ¹²⁵I-192-IgG, respectively.

Retrograde Transport of ¹²⁵I-NGF and ¹²⁵I-192-IgG: Ligands

were injected into the anterior eye chamber of ether-anesthetized experimental animals as previously described (16). The volumes injected were 5 μ l for rats, and 2 μ l for the other species. Animals were obtained as follows: Sprague-Dawley rats (200–225 g) from Chappel Breeders (St. Louis, MO), adult Swiss mice from Harlan Sprague-Dawley, Inc. (Indianapolis, IN), adult gerbils from Tumblebrook Farms, Inc. (West Brookfield, MA), adult hamsters from Engle Laboratory Animals, Inc., (Farmersburg, IN), and guinea pigs from Charles River Breeding Laboratories (Wilmington, MA). Animals were killed by decapitation, and both superior cervical ganglia were dissected and placed in the gamma counter. The radioactivity of the ganglion contralateral to the injected eye provided the background value attributable to spillover of labeled ligand into the systemic circulation. For analysis by SDS PAGE (22), each ganglion was homogenized in 1% SDS.

RESULTS

Solid-Phase Separation Assay

Prior experiments had shown that 192-IgG enhances NGF binding to the NGF receptor (14), but only circumstantial evidence (as described in the introduction) existed that the monoclonal antibody itself actually bound to the NGF receptor. We sought to demonstrate 192-IgG binding to solubilized NGF receptor by the means of a solid-phase separation assay. Radioactive 192-IgG was combined with *n*-octyl glucopyranoside-solubilized membrane proteins from SCG, and then NGF-Sepharose 4B was added to the mixture. If the monoclonal antibody and NGF bind noncompetitively to the NGF receptor, a ternary complex consisting of 125 I-192-IgG, NGF receptor, and NGF-Sepharose 4B will be formed, and the radioactivity would be bound indirectly, but specifically, to the NGF-Sepharose 4B. Bar *b* in Fig. 1 depicts the radioactivity that was associated with the final washed pellet when 10 μ g of solubilized SCG membrane protein was incubated with ~25 fmol of 125 I-192-IgG (50,000 cpm). Bar *a* shows the radioactivity pelleting from a reaction containing no membrane preparation. This amount represents the 125 I-192-IgG that associates nonspecifically with the NGF-Sepharose 4B and therefore defines the background “noise” of this assay. This value from each separate experiment was used to normalize the results from other reaction conditions. We define the signal of the assay as the difference between the values of bars *b* and *a*.

We tested the specificity of the formation of the ternary complex by replacing two of the three components with other molecules, and by displacing either labeled antibody or NGF-Sepharose with excess nonlabeled antibody or soluble NGF, respectively. Substitution of the NGF-Sepharose 4B with an equal volume of Sepharose 4B coupled to the A chain of ricin reduced the radioactivity associated with the pellet to background quantities (bar *c*). This result precluded the possibility that the signal obtained using NGF-Sepharose 4B (bar *b*) resulted from the binding of 125 I-192-IgG to a component of the solubilized SCG membrane preparation that associated nonspecifically either with Sepharose 4B or with any protein molecule linked covalently to Sepharose 4B. Addition of soluble NGF (bar *d*) or nonlabeled 192-IgG (bar *e*), each in 1,000-fold excess of covalently-linked or iodinated species, respectively, eliminated the signal. To show that this decrease in recovered radioactivity represented specific inhibition of the formation of the ternary complex, we added similarly excessive amounts of cytochrome *c* (a protein with similar molecular weight and isoelectric point as NGF, bar *f*) or of a monoclonal IgG directed against the EGF receptor of PC12 cells (bar *g*); these proteins failed to reduce the radioactivity of the pellet. Finally, we demonstrated the requirement for

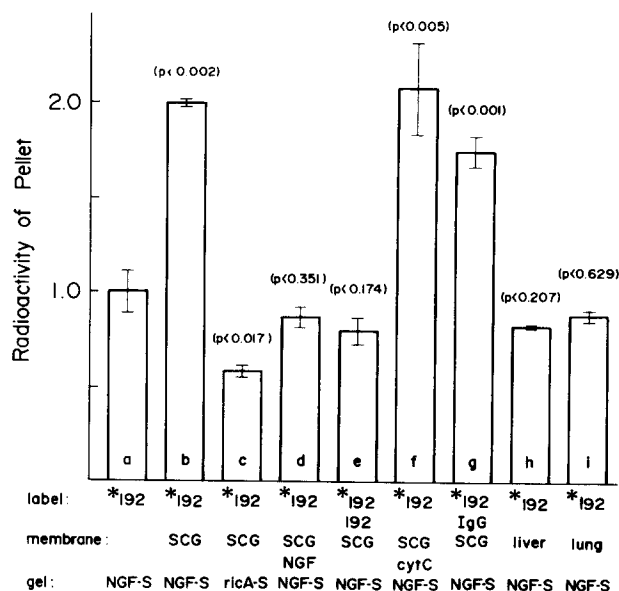


FIGURE 1 Binding of 125 I-192-IgG to solubilized NGF receptor. Radiolabeled 192-IgG was combined with *n*-octyl glucopyranoside-solubilized plasma membrane proteins of rat SCG, liver, or lung, with or without nonlabeled 192-IgG or a control monoclonal IgG, as described in Materials and Methods. After 2 h, NGF-Sepharose 4B or ricin A chain-Sepharose 4B was added, with or without soluble NGF or cytochrome *c*, and the mixtures were allowed to equilibrate for 1 h longer. The microfuge tubes containing the mixtures were then centrifuged, the supernatants aspirated, and the pellets washed and measured for radioactivity. The composition of the mixture is indicated below each bar. The results shown are from two separate experiments. In each experiment three determinations were made for each of the combinations, and the data were normalized to the mean number of cpm obtained from the combination of 125 I-192-IgG and NGF-Sepharose 4B (bar *a*); the actual values of these means were 2,485 cpm and 1,776 cpm. Values plotted are means \pm SEM. One-way analysis of variance with Satterthwaite's correction yielded $P = 0.0015$. Numbers in parentheses are right tail P values derived from Student's t test. *192, 125 I-labeled 192-IgG; NGF-S, NGF-Sepharose 4B; ric A-S, ricin A chain-Sepharose 4B.

the NGF receptor in the formation of the complex: replacing the solubilized SCG membrane preparation with either solubilized rat liver membrane (bar *h*) or solubilized rat lung membrane (bar *i*) abolished the signal.

We also performed analogous experiments using 125 I-NGF, the *n*-octyl glucopyranoside-solubilized SCG membrane proteins, and 192-IgG covalently linked to Sepharose 4B. The results of these solid-phase separation assays (data not shown) were similar to those presented in Fig. 1, and indicated the specific formation of a ternary complex of 125 I-NGF, solubilized NGF receptor, and 192-IgG-Sepharose 4B.

Filtration Binding Assay

To corroborate further the specificity of 192-IgG binding to the NGF receptor, we measured directly the binding of labeled antibody to plasma membrane-enriched fractions prepared from various tissues, known to contain different amounts of NGF receptor, and correlated this to binding of labeled NGF to the same preparations. Fig. 2 shows that only membranes from SCG specifically bound significant amounts of 125 I-NGF and 125 I-192-A. Consistent with results obtained in binding assays using PC12 cells (14), excess 192-IgG in-

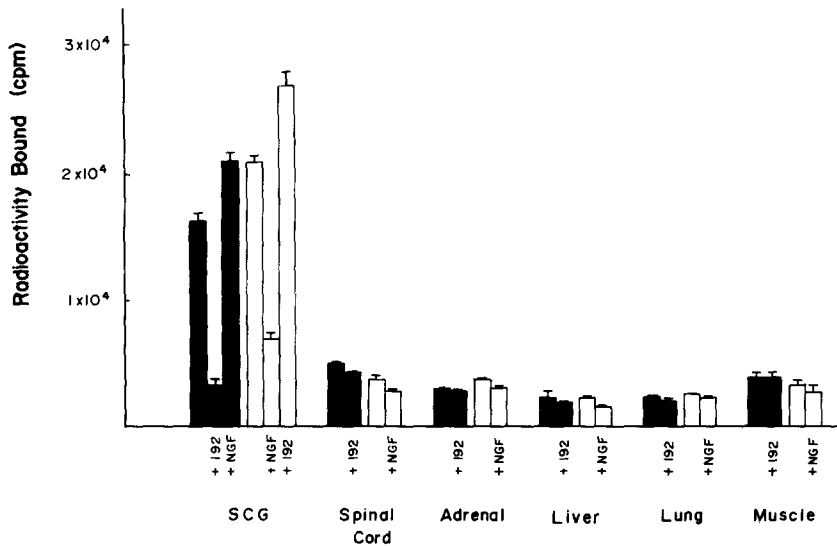


FIGURE 2 Binding of ^{125}I -192-IgG and ^{125}I -NGF to rat tissue membrane preparations. Labeled ligands (^{125}I -192-IgG, 3.7 nM; ^{125}I -NGF, 0.5 nM) were incubated with membrane preparations (0.125 mg/ml of protein) in the absence or presence of excess nonlabeled ligands (192-IgG, 440 nM; NGF, 480 nM) as described in Materials and Methods. After 2 h at 25°C, the mixtures were passed through Millipore filters, and the filters were then washed and measured for radioactivity. The values are the means \pm SEM of triplicate determinations from a single experiment; two other experiments yielded similar results.

creased ^{125}I -NGF binding, and excess NGF increased ^{125}I -IgG binding.

Retrograde Transport

Intraocular injection of ^{125}I -192-IgG into rats resulted in accumulation of radioactivity in the SCG located ipsilateral to the injected eye, whereas no accumulation was detected after injection of ^{125}I -labeled monoclonal IgG against the EGF receptor of PC12 cells (data not shown). We therefore characterized the retrograde transport of labeled 192-IgG in experiments similar to those previously reported for NGF (16). Fig. 3 shows the time course of accumulation of ^{125}I -192-IgG in the SCG. Radioactivity was detectable at 8 h, reached a maximum level at 20 h, and declined rapidly thereafter.

As increasing amounts of ^{125}I -NGF are injected intraocularly, the quantity accumulating in the ipsilateral SCG increases to reach a maximum value. This "functional saturability" is a consequence of the limited capacity in at least one of the stages involved in the overall process of retrograde transport (which include binding of NGF to its receptor, internalization into the neuron, and vectorial translocation through the axon to the soma). If 192-IgG binds to the NGF receptor, and is subsequently internalized and transported by the same mechanisms as is NGF, then the accumulation of antibody in the SCG should share the same saturation properties. Various quantities of nonlabeled 192-IgG were combined with constant amounts of ^{125}I -192-IgG (24.5 pmol, 2.7×10^7 cpm) to achieve samples of different specific radioactivities. These samples were injected into the anterior eye chambers of rats, and the SCG were subsequently dissected. The radioactivity of each SCG was measured separately. Fig. 4 shows that the accumulation of 192-IgG reached a maximum of 1.5 fmol per ganglion, the same value found previously for NGF accumulation (16).

The initial step in the retrograde transport of exogenously administered NGF is the binding of the injected NGF molecule to the NGF receptor. The specificity of this event is inherent to the interaction of a receptor with its particular ligand. Therefore, as shown in Fig. 5 *a*, the co-injection of a 150-fold molar excess of nonlabeled NGF with ^{125}I -NGF eliminated the accumulation of labeled NGF in the SCG. Because the binding of antibody and its target molecule is also specific, nonlabeled 192-IgG should compete with ^{125}I -

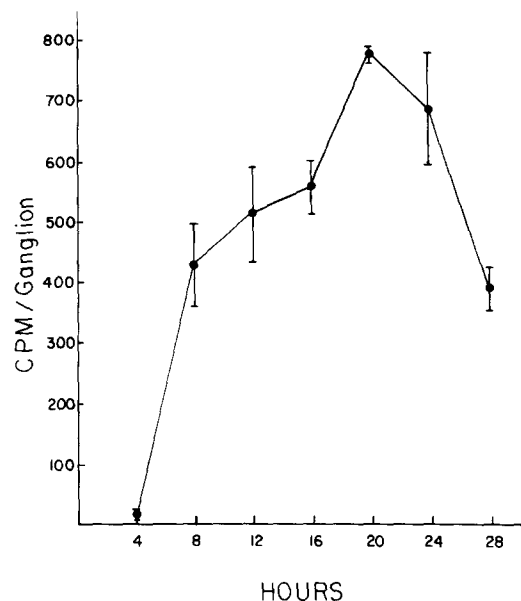


FIGURE 3 Time course of ^{125}I -192-IgG accumulation in the SCG after intraocular injection. Radiolabeled 192-IgG (6×10^6 cpm in 2 μl) was injected into the anterior eye chamber of each rat. At the indicated time intervals both superior cervical ganglia were dissected and the radioactivity of each was measured; from each rat a single value of specifically accumulated radioactivity was calculated by subtracting the cpm associated with the contralateral SCG (<120 cpm) from that of the ipsilateral SCG. Values plotted are the means \pm SEM of 6–7 animals.

192-IgG for binding to the NGF receptor. The data of Fig. 5 demonstrate this competition: a 35-fold molar excess of nonlabeled antibody co-injected with ^{125}I -192-IgG reduced the accumulation of radioactivity in the SCG. We were unable to obtain a greater ratio of nonlabeled to labeled 192-IgG because of the limited solubility of 192-IgG (7.5 mg/ml) and the small volume that can be injected (5 μl).

Consistent with the data of the *in vitro* binding assays (14), NGF and 192-IgG failed to compete with each other for retrograde transport; there appears to have been, in fact, mutual enhancement of accumulation (Fig. 5, *a* and *b*). These results reflect the fact that NGF and 192-IgG can bind non-competitively to the NGF receptor as the first step in retro-

grade transport. Ability to bind simultaneously to the receptor protein should allow co-transport of NGF and 192-4, and therefore injection of ^{125}I -NGF and ^{125}I -192-IgG together should lead to accumulation of both in the SCG. The results shown in Fig. 5c and Fig. 6 support this. The amount of radioactivity that accumulated when ^{125}I -NGF and ^{125}I -192-IgG were injected together is equal to the sum of the quantities obtained when they were injected alone (Fig. 5c). The autoradiograph (Fig. 6) shows that intact ^{125}I -192-IgG and ^{125}I -NGF were both present in the SCG when these radiolabeled ligands were co-injected, thus verifying that they were transported simultaneously. The extraneous bands of lanes 4, 5, and 6 are degradation products of 192-IgG, presumably formed in the SCG.

The retrograde transport of 192-IgG appears to be species specific (Table I). There was no accumulation of radioactivity following intraocular injection of ^{125}I -192-IgG in mice, gerbils, hamsters, and guinea pigs. Separate injections of ^{125}I -NGF did lead to the accumulation of label in the ipsilateral SCG

of these species, verifying that the injection technique was sound.

DISCUSSION

The initial description of monoclonal antibody 192-IgG established its effect of increasing the affinity of NGF binding to the NGF receptor of PC12 cells, and demonstrated that 192-IgG binds to the same number of sites as does NGF and that it does not bind NGF (14). In this paper we have confirmed the specific binding of 192-IgG to the NGF receptor, both in vitro and in vivo. Results of the solid-phase separation assay demonstrated that 192-IgG can form a ternary complex with solubilized NGF receptor and NGF. The result that soluble NGF, but not soluble cytochrome *c*, blocked the association of ^{125}I -192-IgG with the NGF-Sepharose showed that this association involved the specific interaction of NGF with its receptor. The competition by nonlabeled 192-IgG, but not by an irrelevant monoclonal IgG, attested to the specificity of the interaction of 192-IgG with the NGF-binding moiety. The fact that solubilized plasma membrane protein from rat SCG, but not liver or lung, was necessary and sufficient for the association of ^{125}I -192-IgG with the NGF-Sepharose 4B pellet argues further that it was the NGF receptor which linked these two species. The filtration binding assay showed that the 192-IgG binds to an epitope that is present only in certain tissues; the distribution of this epitope is consistent with that expected of the NGF receptor.

The accumulation within the neuronal cell bodies (in the SCG) of a protein administered to the nerve termini (at the iris) requires the following processes: binding to receptors, internalization, and active retrograde transport. The retrograde axonal transport of 192-IgG displayed a time course similar to that described for NGF (16). In particular, 192-IgG accumulation was detectable between 4 and 8 h after intraocular injection, indicating the same rate of transport (~ 3 mm/hour, assuming a 2-cm distance between the iris and the SCG) as that of NGF. Likewise, the value of maximum 192-IgG accumulation, 1.5 fmol, equals the reported value of maximum NGF accumulation (16). These results are consistent with the hypothesis that 192-IgG binds to the NGF

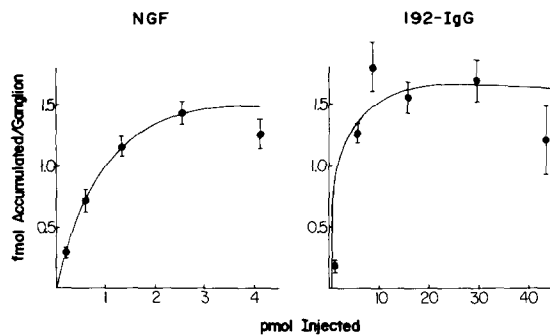


FIGURE 4 Concentration dependence of NGF or 192-IgG accumulation in the SCG. Labeled ligand (^{125}I -NGF or ^{125}I -192-IgG) was combined with different amounts of nonlabeled ligand (NGF or 192-IgG, respectively) to attain solutions of particular specific activities. 5 μl of solution was injected into the anterior eye chamber of each rat. After 16 h both SCG were dissected and the radioactivity of each was measured. The value of the contralateral SCG was subtracted from that of the ipsilateral, and this difference was converted to molar values of injected ligand. Plotted values are the means \pm SEM of 6-7 rats. The results with NGF are recalculated from a previous study (16).

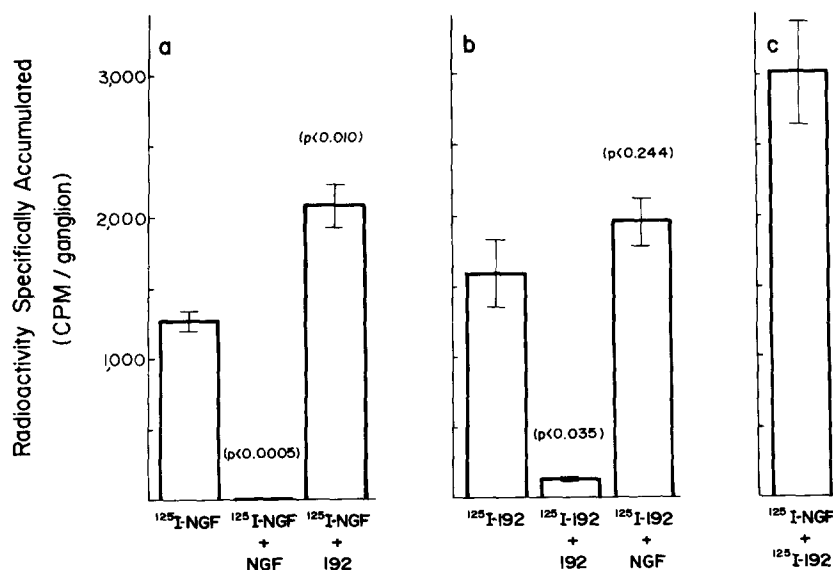


FIGURE 5 Specificity of retrograde transport and co-transport of NGF and 192-IgG. Radio-labeled NGF (2×10^6 cpm, 2 pmol) or 192-IgG (8×10^6 cpm, 5 pmol) was injected alone, or with nonlabeled NGF (500 pmol) or 192-IgG (160 pmol), or together, as indicated below the graphs. The volume of each injection was 5 μl . After 16 h both SCG were dissected and the radioactivity of each was measured. The value of the contralateral SCG was subtracted from that of the ipsilateral for each pair. Values represent the mean \pm SEM of 5-7 rats. One-way analysis of variance with Satterthwaite's correction yielded $P = 0.0001$ for the data of a, and $P = 0.0003$ for the data of b. Numbers in parentheses indicate right tail P values derived from Student's t test.

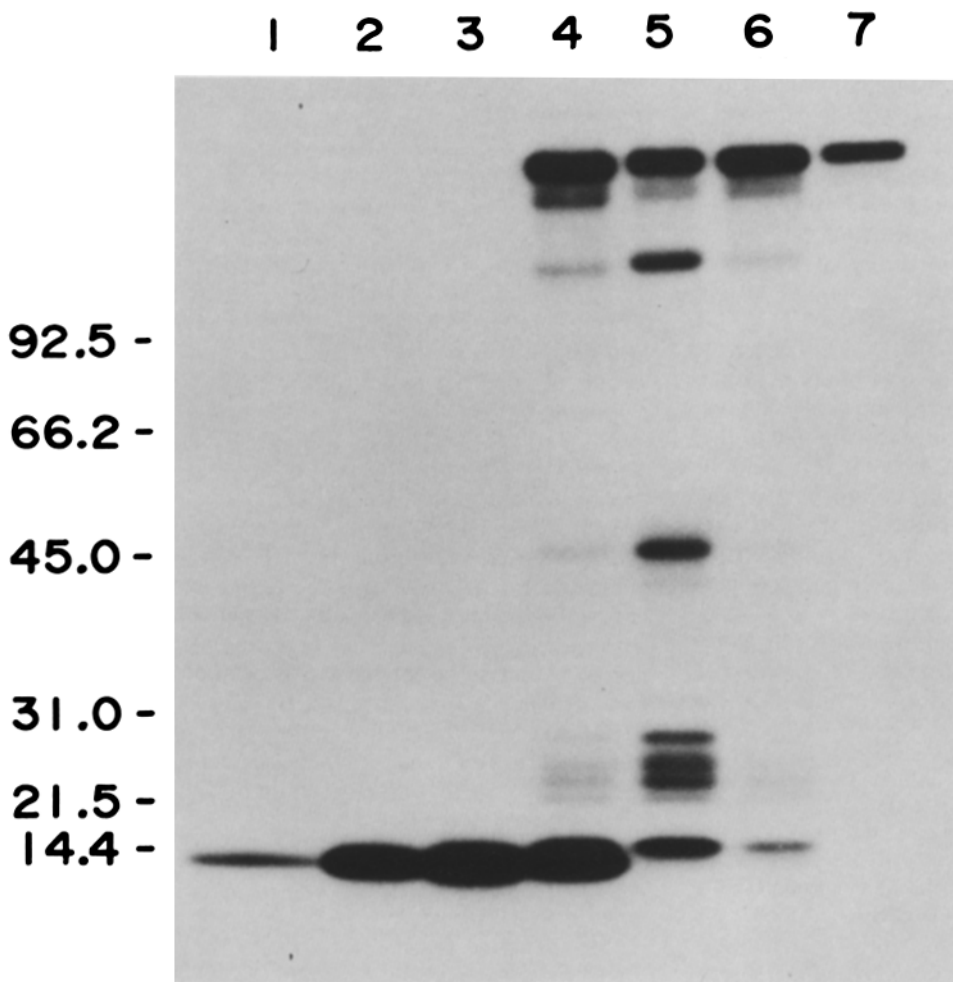


FIGURE 6 SDS PAGE and autoradiography of SCG with accumulated ^{125}I -NGF and ^{125}I -192-IgG. The ipsilateral SCG of rats injected intraocularly with radio-labeled ligands (see Fig. 5) were homogenized in 1% SDS and electrophoresed on a 7–20% exponential gradient gel. The gel was stained with Coomassie Brilliant Blue R, destained, dried, and exposed to Kodak X-Omat film for 4 d. Lane 1, ^{125}I -NGF; lane 2, SCG of rat injected with ^{125}I -NGF; lane 3, SCG of rat injected with ^{125}I -NGF and nonlabeled 192-IgG; lane 4, SCG of rat injected with ^{125}I -NGF and ^{125}I -192-IgG; lane 5, SCG of rat injected with ^{125}I -192-IgG and nonlabeled NGF; lane 6, SCG of rat injected with ^{125}I -192-IgG; lane 7, ^{125}I -192-IgG. The molecular weight standards were phosphorylase B (92,500); bovine serum albumin (66,200); ovalbumin (45,000); carbonic anhydrase (31,000); soybean trypsin inhibitor (21,500); lysozyme (14,400).

TABLE I. Comparison of 192-IgG Retrograde Transport in Rat and Non-Rat Species

Species	Specific accumulation* of ^{125}I -192-IgG in ipsilateral SCG	Specific accumulation of ^{125}I -NGF in ipsilateral SCG
	cpm	cpm
Rat	1,588.5 \pm 240.8 (4)	1,267.8 \pm 69.2 (6)
Mouse	3.0 \pm 4.1 (4)	—
Gerbil	0.6 \pm 4.1 (7)	464 (2)
Hamster	-0.3 \pm 3.0 (8)	475 (2)
Guinea pig	42.5 \pm 35.5 (4)	2,835 (2)

2 μl of ^{125}I -192-IgG (2×10^6 cpm) or of ^{125}I -NGF (2×10^6 cpm) was injected into the anterior eye chamber of each animal. The SCG were dissected after 16 h for rats and guinea pigs, 8 h for gerbils and hamsters, and 6 h for mice.

* Specific accumulation is defined as the difference: (counts per minute of ipsilateral SCG) - (counts per minute of contralateral SCG). Values are means \pm SEM; values in parentheses indicate the number of experimental animals.

receptor, becomes internalized, and is transported by the same mechanisms as is NGF. The internalization and retrograde axonal transport of 192-IgG in the presence of only the minute quantity of endogenous NGF (i.e., without any experimental administration of NGF) raises the possibility that the NGF receptor is continuously internalized and retrogradely transported, even in the absence of bound NGF. Such a constitutive transport of the NGF receptor is also suggested by the observation that ^{125}I -labeled NGF is specifically bound and

retrogradely transported from the crush site of axotomized sciatic nerve in rats (24, 28) and newts (24). Continuous internalization of receptor proteins independent of ligand binding has been proposed for at least one other system, that of the low density lipoprotein receptor of human fibroblasts (29).

The retrograde transport of 192-IgG therefore can serve as an in vivo binding assay for the monoclonal antibody. We interpret the lack of retrograde transport of 192-IgG in mice, gerbils, hamsters, and guinea pigs as a manifestation of the species specificity of the monoclonal antibody (i.e., 192-IgG binds to an epitope found only on the rat NGF receptor). This specificity was also observed in results of the filtration binding assay in which ^{125}I -labeled 192-IgG did not bind specifically to mouse SCG membrane preparations (data not shown).

The binding characteristics of 192-IgG are similar to other receptor-directed antibodies, both monoclonal and polyclonal, that bind to determinants outside of the ligand-binding sites. For example, Richert et al. have described a monoclonal IgG3 directed against the EGF receptor of A431 cells that fails to inhibit EGF binding (30), and Schreiber et al. have documented a monoclonal IgG2a that immunoprecipitates the EGF receptor but does not interfere with its binding of EGF (31). Jacobs et al. (32) and Grunfeld (33) have shown that polyclonal antibodies directed against the insulin receptor exert biological effects on the receptor without blocking in-

sulin binding. The noncompetitive binding of 192-IgG and NGF to the NGF receptor makes this monoclonal antibody uniquely suitable for probing the NGF receptor without inhibiting the interaction of the receptor with its hormone ligand. Because of this noncompetitive nature of 192-IgG and NGF binding, experimental perturbation of the biology of neurons using 192-IgG would implicate mechanisms involving the receptor directly, exclusive of its interaction with NGF. In addition, it should be possible to use 192-IgG as a specific histological ligand for the NGF receptor irrespective of the NGF occupation state of the receptor.

The specific retrograde transport of 192-IgG confirms that it can bind to the NGF receptor *in vivo*. This monoclonal antibody can therefore be used as a specific probe for the NGF receptor, both in the intact rat to study the role of the receptor in mediating the biological effects of NGF, and in tissues to detect immunohistochemically the presence of the receptor. It can also serve to target particular molecules to NGF receptor-bearing cells and thereby make possible experiments to manipulate these neurons specifically. For example, a hybrid molecule of 192-IgG linked to the toxic ricin A chain would bind to an NGF receptor-containing cell via the antibody moiety, then, following internalization, kill the cell via inhibition of protein synthesis by the ricin A chain.

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REFERENCES

1. Levi-Montalcini, R., and P. U. Angeletti. 1968. Nerve growth factor. *Physiol. Rev.* 48:534-569.
2. Thoenen, H., and Y.-A. Barde. 1980. Physiology of nerve growth factor. *Physiol. Rev.* 60:1284-1335.
3. Korsching, S., and H. Thoenen. 1983. Nerve growth factor in sympathetic ganglia and corresponding target organs of the rat: correlation with density of sympathetic innervation. *Proc. Natl. Acad. Sci. USA.* 80:3513-3516.
4. Hendry, I. A., K. Stöckel, H. Thoenen, and L. L. Iverson. 1974. The retrograde axonal transport of nerve growth factor. *Brain Res.* 68:103-121.
5. Korsching, S., and H. Thoenen. 1983. Quantitative demonstration of the retrograde axonal transport of endogenous nerve growth factor. *Neurosci. Lett.* 39:1-4.
6. Palmatier, M. A., B. K. Hartman, and E. M. Johnson, Jr. 1984. Demonstration of retrogradely transported endogenous nerve growth factor in axons of sympathetic neurons. *J. Neurosci.* 4:751-756.
7. Kahn, C. R., K. L. Baird, D. B. Jarrett, and J. S. Flier. 1978. Direct demonstration that receptor crosslinking or aggregation is important in insulin action. *Proc. Natl. Acad. Sci. USA.* 75:4209-4213.
8. Schreiber, A. B., I. Lax, Y. Yarden, Z. Eshhar, and J. Schlessinger. 1981. Monoclonal antibodies against receptor for epidermal growth factor induce early and delayed effects of epidermal growth factor. *Proc. Natl. Acad. Sci. USA.* 78:7535-7539.
9. Banerjee, S. P., S. H. Synder, P. Cuatrecasas, and L. A. Greene. 1973. Binding of nerve growth factor receptor in sympathetic ganglia. *Proc. Natl. Acad. Sci. USA.* 70:2519-2523.
10. Frazier, W. A., L. F. Boyd, and R. A. Bradshaw. 1974. Properties of the specific binding of ¹²⁵I-nerve growth factor to responsive peripheral neurons. *J. Biol. Chem.* 249:5513-5519.
11. Fabricant, R. N., J. E. DeLarco, and G. J. Todaro. 1977. Nerve growth factor receptors on human melanoma cells in culture. *Proc. Natl. Acad. Sci. USA.* 74:565-569.
12. Greene, L. A., and A. S. Tischler. 1976. Establishment of a noradrenergic clonal line of rat adrenal pheochromocytoma cells which respond to nerve growth factor. *Proc. Natl. Acad. Sci. USA.* 73:2424-2428.
13. Puma, P., S. E. Busser, L. Watson, D. J. Kelleher, and G. L. Johnson. 1983. Purification of the receptor for nerve growth factor from A875 melanoma cells by affinity chromatography. *J. Biol. Chem.* 258:3370-3375.
14. Chandler, C. E., L. M. Parsons, M. Hosang, and E. M. Shooter. 1984. A monoclonal antibody modulates the interaction of nerve growth factor with PC12 cells. *J. Biol. Chem.* 259:6882-6889.
15. Fernandez-Pol, J. A., D. J. Klos, and P. D. Hamilton. 1982. Identification of nerve growth factor receptor proteins in mouse melanoma cell membranes after electrophoretic transfer from gels to diazo-paper. *Biochemistry International.* 5:213-217.
16. Johnson, E. M., Jr., R. Y. Andres, and R. A. Bradshaw. 1978. Characterization of the retrograde transport of nerve growth factor (NGF) using high specific activity [¹²⁵I]NGF. *Brain Res.* 150:319-331.
17. Taniuchi, M., P. T. Manning, C. Chandler, L. Chandler, and E. M. Johnson, Jr. 1984. *In vivo* studies of an NGF receptor-interactive monoclonal antibody. *Fed. Proc.* 43:504. (Abstr.)
18. Bocchini, V., and P. U. Angeletti. 1969. The nerve growth factor: purification as a 30,000 molecular weight protein. *Proc. Natl. Acad. Sci. USA.* 64:787-794.
19. Levy, H. B., and H. A. Sober. 1960. A simple chromatographic method for preparation of gamma globulin. *Proc. Soc. Exp. Biol. Med.* 103:250-253.
20. Nicholson, G. L., and J. Blanstien. 1972. The interaction of ricinus communis agglutinin with normal and tumor cell surfaces. *Biochem. Biophys. Acta.* 266:543-547.
21. Simmons, B. M., and J. H. Russell. 1985. A single affinity column step method for the purification of ricin toxin from castor beans. *Anal. Biochem.* 146:206-210.
22. Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature (Lond.).* 227:680-685.
23. Marchalonis, J. J. 1969. An enzyme method for the trace iodination of immunoglobulins and other proteins. *Biochem. J.* 113:229-305.
24. Yip, H. K., and E. M. Johnson, Jr. 1983. Retrograde transport of nerve growth factor in lesioned goldfish retinal ganglion cells. *J. Neurosci.* 3:2172-2182.
25. Costrini, N. Y., and R. A. Bradshaw. 1979. Binding characteristics and apparent molecular size of detergent-solubilized nerve growth factor receptor of sympathetic ganglia. *Proc. Natl. Acad. Sci. USA.* 76:3242-3245.
26. Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall. 1951. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* 193:265-275.
27. Axén, R., J. Porath, and S. Ernback. 1967. Chemical coupling of peptides and proteins to polysaccharides by means of cyanogen halides. *Nature (Lond.).* 214:1302-1304.
28. Richardson, P. M., and R. J. Riopelle. 1984. Uptake of nerve growth factor along peripheral and spinal axons of primary sensory neurons. *J. Neurosci.* 4:1683-1689.
29. Anderson, R. G. W., M. Brown, and J. L. Goldstein. 1977. Role of the coated endocytic vesicle in the uptake of receptor-bound low density lipoprotein in human fibroblasts. *Cell.* 10:351-364.
30. Richert, N. D., M. C. Willingham, and I. Pastan. 1983. Epidermal growth factor receptor. *J. Biol. Chem.* 258:8902-8907.
31. Schreiber, A. B., T. A. Libermann, I. Lax, Y. Yarden, and J. Schlessinger. 1983. Biological role of epidermal growth factor-receptor clustering. *J. Biol. Chem.* 258:846-853.
32. Jacobs, S., K. Chang, and P. Cuatrecasas. 1978. Antibodies to purified insulin receptor have insulin-like activity. *Science (Wash. DC).* 200:1283-1284.
33. Grunfeld, C. 1984. Antibody against the insulin receptor causes disappearance of insulin receptors in 3T3-L1 cells: a possible explanation of antibody-induced insulin resistance. *Proc. Natl. Acad. Sci. USA.* 81:2508-2511.