Journal of Cellular Biochemistry 27:121–132 (1985)

Production of a Monoclonal Antibody Directed Against the Nerve Growth Factor Receptor From Sympathetic Membranes

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Spleen cells from BALB/c mice immunized with a plasma membrane-enriched fraction from rabbit sympathetic ganglia were fused with the mouse myeloma NS1. A hybrid clone was obtained that produced monoclonal antibody directed against the receptor for nerve growth factor (NGF). The antibody, identified as IgG, was able to immunoprecipitate solubilized NGF receptor in the presence or absence of bound NGF. The antibody bound specifically to sympathetic membranes with high affinity but did not affect the binding of ^{125}I-NGF to its receptor in sympathetic or sensory neurons or PC12 cells.

Key words: growth factor receptors, monoclonal antibodies, nerve growth factor

Characterization of the cell surface receptor for nerve growth factor (NGF) has been restricted primarily to measurement of kinetic and equilibrium parameters derived from the specific binding of ^{125}I-NGF to responsive tissues and correlation of this binding with biological activity [1]. Determination of the molecular structure of the receptor has been hindered by the fact that, like many other growth factor receptors, the NGF receptor represents a very small percentage of total cellular or even cell surface proteins. Thus purification of even analytical amounts of receptor for direct physical studies is difficult. It has been necessary, therefore, to utilize alternatives to classical techniques for the determination of receptor structure. Covalent cross linking of ^{125}I-NGF to its receptor using the photochemical reagent N-hydroxysuccinimidyl-p-azidobenzoate (HSAB) revealed a molecular weight of 130 kDa, with a 100 kDa derivative, for the NGF receptor in rabbit sympathetic membranes [2], with similar values found subsequently for chick sensory ganglia and rat

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Received March 8, 1984; accepted September 28, 1984.

PC12 cells [3,4]. This is in good agreement with the value determined by Costrini et al [5] from hydrodynamic measurements of the detergent-extracted receptor from sympathetic ganglia and those obtained by Fernandez-Pol et al [6] by immunochemical staining of electrophoretic blots from mouse melanoma cells.

Recently, a highly purified preparation of NGF receptor has been obtained from a human melanoma cell line (A875) that possesses large numbers of receptor sites [7]. This preparation was found to contain two peptides of M_r 85,000 and 200,000. Grob et al [8], also using covalent cross linking agents, found similar results with A875 cells and an M_r of only 87,000 species in PC12 cells. In their hands, the 200 kDa species from human melanoma cells was quite transient, an observation similar to that reported by Costrini et al [5] for rabbit sympathetic neurons. The relationship of these various receptor polypeptides from different species and tissues remains to be elucidated.

The advent of advanced immunological techniques, notably hybridoma and antiidiotype technologies, has greatly augmented available methodologies by facilitating the production of antisera directed against hormone receptors without prior purification. We report here the generation of a hybrid cell line that produces a monoclonal antibody that recognizes the NGF receptor in sympathetic tissue. This antibody has been purified and partially characterized in anticipation of its use as a structural and physiological probe of NGF receptor function.

MATERIALS AND METHODS

Preparation of NGF and ^{125}I-NGF

2.5S NGF was prepared from adult male mouse submaxillary glands (Pel-Freez) as described by Bocchini and Angeletti [9]. ^{125}I-NGF was prepared by the method of Bolton and Hunter [10]. ^{125}I-3-(4-hydroxyphenyl)propionyl-N-hydroxysuccinimide (^{125}I-labeled Bolton-Hunter reagent; New England Nuclear, Boston, MA) was utilized to label NGF by a modification of the method of Banerjee et al [11]. Briefly, 2 mCi of ^{125}I-Bolton Hunter reagent (2,000 Ci/mmol) was incubated with 0.4 nmol of NGF in 5-20 μl of cold 0.1 M borate buffer, pH 8.8. After 10 min at 4°C, the reaction was stopped by the addition of 100 μl of 0.5 M glycine in 0.1 borate buffer, pH 8.5. After 5 min at 25°C, 300 μl of 6 M guanidine hydrocholoride was added and the reaction mixture was applied to a PD-10 column (Pharmacia) and eluted with Hank's balanced salt solution (HBSS, Gibco, Grand Island, NY)/10 mM Hepes medium, pH 7.5, containing 0.2% gelatin. Prior to use, the ^{125}I-NGF was further chromatographed on a column (2 × 17.5 cm) of Bio-Gel P-100 (Bio-Rad), which was eluted with HBSS/10 mM Hepes buffer, pH 7.5, containing 2 mg/ml bovine serum albumin (BSA, radioimmunoassay grade, Sigma, St. Louis, MO). Incorporation of ^{125}I with the Bolton-Hunter reagent was 50–75%; specific activity was 4,000–7,000 dpm/fmol monomer (140–240 μCi/μg).

Alternatively, NGF was radioiodinated by a modification of the lactoperoxidase procedure described by Tait et al [12]. Lactoperoxidase-catalyzed iodination was carried out in 20 mM sodium phosphate buffer, pH 6.0. NGF (2-5 μg in H_2O) was added to 1 mCi Na ^{125}I (100 mCi/ml, pH 7–11, Amersham, Amersham, UK) and 0.36 μg bovine lactoperoxidase (Sigma). The reaction was initiated by addition of 2 μl H_2O_2 (500 μM in H_2O) and allowed to proceed for 3 min at 24°C. A second aliquot of H_2O_2 was added and the reaction was continued for an additional 3 min.

The reaction was quenched by the addition of 100 μl of a solution of 1 M NaCl, 100 mM NaI, 50 mM sodium phosphate, 1 mM NaN_3, 2 mg/ml BSA, 1 mg/ml protamine sulfate, pH 7.5. The mixture was chromatographed on a column (1.5 × 18 cm) of Bio-Gel P-100 in HBSS/10 mM Hepes buffer, pH 7.5 containing 2 mg/ml BSA. Incorporation of ^{125}I was 50–70%; specific activity was 2,000–4,000 dpm/fmol monomer (70–140 μCi/μg). ^{125}I-NGF was stored at −20°C in 10 mg/ml BSA and filtered through Acrodisc 0.2 μm filters (Gelman) prior to use.

Preparation and Solubilization of Microsomes

Superior cervical ganglia from mature rabbits, obtained frozen from Pel-Freez (Rogers, AR), were used as the source of NGF receptor. A plasma membrane-enriched microsomal fraction was prepared according to a modification of the method of Banerjee et al [13], which has been described in detail elsewhere [14]. Microsomes were solubilized in HBSS/10 mM Hepes buffer containing 1% Triton X-100 (pH 7.5) and incubated on ice for 45 min. Insoluble material was removed by centrifugation at 100,000g for 1 hr. The supernatant was diluted eightfold with HBSS/10 mM Hepes, pH 7.5, to adjust the final detergent concentration to 0.12%. Protein concentration was determined according to the Lowry method [15] as modified by Markwell et al [16].

Dodecyl Sulfate Polyacrylamide Gel Electrophoresis

Samples were solubilized in 50 mM Tris-Cl buffer (pH 8.6) containing 1% sodium dodecyl sulfate and 5% 2-mercaptoethanol unless otherwise indicated. Electrophoresis was performed according to Laemmli [17]. Molecular weight standards (Bio-Rad Laboratories) were bovine serum albumin (M_r 66,000), ovalbumin (M_r 45,000), carbonic anhydrase (M_r 31,000), soybean trypsin inhibitor (M_r 21,000), and lysozyme (M_r 14,400).

Cell Lines and Media

The parental myeloma line P3-NSI/1-Ag4-1 (NS1) [18] is a nonsecreting derivative of the MOPC-21 myeloma [19]. The NS1 line produces light chains (κ), which are secreted only in the hybrids in association with spleen cell-specific heavy chains. This line and all derived hybrids were maintained in Dulbecco's modified Eagle's medium (high glucose, high bicarbonate) (Center for Basic Cancer Research, Washington University, St. Louis, MO) supplemented with 5% calf serum (K.C. Biologicals, Kansas City, MO), 10% agammaglobulin horse serum, penicillin (100 U/ml), and streptomycin (100 μg/ml, all from Gibco), designated as "complete medium." HAT medium was supplemented with hypoxanthine (14 μg/ml), aminopterin (.176 μg/ml), and thymidine (3 μg/ml, all from Sigma). HT medium contained hypoxanthine (14 μg/ml) and thymidine (3 μg/ml).

PC12 cells (originally obtained from Dr. Lloyd Greene), cultured in RPMI 1640 supplemented with 5% fetal calf serum and 10% horse serum (Gibco), were generously provided by Chris Powers.

Immunization

Female BALB/c mice (Cumberland View Farms) were injected intraperitoneally with 250–300 μg of rabbit SCG crude microsomes in HBSS/10 mM Hepes, pH 7.5, containing no adjuvant. Two further injections were given at monthly intervals. Three days after the third injection spleens were removed for fusion.

Fusion and Cloning of Hybrids

Spleen cells ($2–3 \times 10^8$) and myeloma cells (10^7) were fused using polyethylene glycol 1500 (Aldrich Chemical Co., Milwaukee, WI) by the method of Galfre et al [20]. Immediately after fusion cells were distributed into 24 wells (2 ml/well, Costar 3524). After 24, 48, and 72 hr, half the medium was replaced by HT medium [21]; medium was replaced only as needed for the next 2 weeks. Supernatants were first screened for antibody activity after 2 weeks. Subsequently, cultures were adapted first to HT medium then to complete medium over approximately 2 weeks. Positive wells were expanded into larger cultures and frozen as soon as possible in liquid nitrogen in a 50% solution of cryoprotective medium (M.A. Bioproducts, Walkersville, MD) in complete medium. Single clones were obtained by twice plating cultures at 1 cell/well in 96-well plates (Costar 3596) on a feeder layer of peritoneal macrophages obtained from Swiss-Webster mice. In a total of 12 fusions, two spleens yielded positive hybrids (although some antireceptor antibody-producing clones may have gone undected due to the presence of interfering serum components, as discussed below).

Production of Ascites

BALB/c mice were primed by intraperitoneal injection of 0.5 ml of pristane (2,5,10,14-tetramethylpentadecane, Aldrich Chemical Co.); 1 to 2 weeks later $10^6–10^7$ hybrid cells were injected intraperitoneally. Ascites fluid was collected within 2 to 4 weeks (2–10 ml/mouse); cells were removed by centrifugation in a Sorvall GLC-2 (2,000 rpm, 5 min) and ascites fluid was frozed in 1 ml aliquots at −70°C. Before use, the ascites fluid was partially purified using a 50% ammonium sulfate precipitation, primarily to remove serum albumin (AS-ascites).

Indirect Immunoprecipitation Assay

Solubilized SCG plasma membrane-enriched microsomes (10–20 μg) were incubated at 4°C for 16 hr with hybridoma supernatants or ascites fluid in 25 mM Hepes (pH 7.5). Antigen-antibody complexes were immunoprecipitated by addition of goat antimouse immunoglobulin (Gateway Immunosera, St. Louis, MO) followed by incubation of 4°C for 4 hr and centrifugation in a Beckman microfuge (4 min). Supernatants were assayed in duplicate for ^{125}I-NGF binding activity by incubating them with 0.2 nM ^{125}I-NGF (prepared by the Bolton-Hunter method) and 5 mg/ml BSA in a final volume of 200 μl at 24°C. After 90 min, samples were placed on ice in the cold room and 0.5 ml ice-cold 0.125% gammaglobulin and 0.5 ml ice-cold 24% polyethylene glycol 6000 (PEG, Sigma), both in HBSS/10 mM Hepes, were added sequentially. After 25 min on ice, samples were centrifuged at 4°C for 20 min at 2,200 rpm (International Centrifuge). Pellets were washed with ice-cold 10% PEG in HBSS/10 mM Hepes and counted in a Beckman 300 gamma counter.

Direct Immunoprecipitation Assay

Solubilized microsomes (15 μg) were incubated with ^{125}I-NGF (0.5 nM) in HBSS/10 mM Hepes buffer, pH 7.5, containing 5 mg/ml BSA in the presence of absence of 2 μM unlabeled NGF for 2 hr at 24°C. Purified antibody in HBSS/10 mM Hepes was added and the solution incubated for 16 hr at 4°C. Complexes were immunoprecipitated by the addition of a slight excess of goat antimouse immunoglobulins followed by incubation for 4 hr at 4°C and centrifugation in a Beckman microfuge (4 min). Pellets were washed with HBSS/10 mM Hepes and counted.

Partial Purification of IgG on Sepharose 6B

Ascites fluid, diluted with an equal volume of PBS, pH 7.5, or hybridoma-conditioned media was stirred for 1 hr at 24°C with an equal volume of saturated ammonium sulfate. The resulting suspension was centrifuged in a Sorvall centrifuge (4,000g, 10 min, 4°C) and the pellet was washed with 50% ammonium sulfate. The pellet was then dissolved in 1 ml H_2O, applied to a 1.5 × 43 cm column of Sepharose 6B (Sigma), and eluted with HBSS/10 mM Hepes, pH 7.5, at 4°C. Pooled fractions were dialyzed against H_2O and lyophilized.

Purification of IgG on DEAE Cellulose

IgG was purified according to the procedure of Levy and Sober [22]. Whole ascites fluid was diluted with an equal volume of 35 mM sodium phosphate, pH 6.3, adjusted to pH 6.3 with 0.2 M NaH_2PO_4, and applied at 24°C to a column (1.5 × 8 cm) of DEAE-cellulose (Sigma) equilibrated with 17.5 mM sodium phosphate, pH 6.3. IgG was collected in the breakthrough fractions, and the remaining protein was eluted with 0.4 M sodium phosphate, pH 5.3, containing 2 M NaCl. The breakdown fractions were dialyzed overnight at 4°C against HBSS/10 mM Hepes, pH 7.5.

Preparation of ^{125}I-Labeled Monoclonal Antibody

Ten micrograms of purified or partially purified immunoglobulin was iodinated using the lactoperoxidase procedure described for radioioidination of NGF [12]. Iodinated antibody was separated from free iodine using a PD-10 column (Pharmacia), eluted with HBSS/10 mM Hepes, pH 7.5, containing 2 mg/ml BSA. Pooled fractions containing the iodinated antibody were stored at 4°C or −20°C in HBSS/10 mM Hepes containing 10 mg/ml BSA.

Binding of ^{125}I Antibody to SCG Microsomes

Microsomes (5 μg) were incubated with ^{125}I-antibody in the presence or absence of 0.3 μM unlabeled antibody in HBSS/10 mM Hepes containing 5 mg/ml BSA. After 1.5 hr at 24°C samples were diluted, centrifuged (2,200 rpm, 20 min), and washed with cold HBSS/10 mM Hepes. Pellets were counted in a gamma counter.

^{125}I-NGF Binding Competition

Microsomes (5 μg), chick DRG neurons (10^4 cell/tube), or PC12 cells were preincubated with increasing concentrations of purified antibody (50 ng/ml to 50 μg/ml) in HBSS/10 mM Hepes containing 5 mg/ml BSA for 2 hr at 24°C. ^{125}I-NGF (0.2 nM final concentration) was then added and the mixture was incubated for an additional 2 hr at 24°C. Following incubation, samples were diluted, centrifuged (2,200 rpm, 20 min), washed once with ice-cold HBSS/10 mM Hepes, and counted. Alternatively, cells or microsomes were incubated simultaneously with antibody and ^{125}I-NGF for 1.5 hr at 24°C before collecting and counting.

RESULTS

Identification of Antireceptor Monoclonal Antibody

Primary fusion products and clones were screened using an indirect immunoprecipitation assay. In this assay, crude soluble receptor was incubated with conditioned media or ascites fluid, and antigen-antibody complexes were immunoprecipitated with

goat antimouse immunoglobulin. The immunoprecipitation supernatant was then measured for ^{125}I-NGF binding, reflecting unprecipitated NGF receptor. A positive response to this assay is indicated by a decrease in ^{125}I-NGF binding activity in the immunoprecipitation supernatant. Four wells of the original 24 plated from a single fusion were positive by this criterion. These wells were cloned twice and a total of four single positive clones were cultured and frozen. One clone, G91E4, was the slowest growing and apparently the most strongly positive; this line was expanded in culture and used in the production of ascites.

In initial indirect immunoprecipitation assays, it was apparent that ascites and serum-containing conditioned media contained an NGF-binding component that interfered with the detection of antireceptor antibody. In the assay, the soluble receptor activity was decreased by no more than 20% by all hybrid cell supernatants. Moreover, when media conditioned by the parent line NSI or 10 mM HBSS/Hepes/0.2% BSA alone were compared as controls for the assay, apparent receptor binding was significantly higher in the conditioned media than HBSS/Hepes buffer. When the assay was carried out using ascites fluid (50% ammonium sulfate fraction as a source of antibody), PEG-precipitable radioactivity was increased as much as fourfold over the control buffer value instead of decreasing due to immunoprecipitation of soluble receptor by ascites antibody. At the highest dilutions of ascites, the NGF binding activity was no longer apparent, and PEG-precipitable radioactivity was equivalent to that observed in the absence of ascites or hybridoma-conditioned media (Fig. 1, upper panel). This evidence indicated that ascites contained an NGF binding protein, independent of an antireceptor antibody, which was not precipitated by antireceptor antibody or goat antimouse immunoglobulin and therefore remained in the immunoprecipitation supernatant. This component was able to bind ^{125}I-NGF and be precipitated by PEG, thus resembling soluble NGF receptor. In order to correct for the NGF binding component, each indirect immunoprecipitation assay was carried out in the presence or absence of soluble receptor (Fig. 1, upper panel). Subtraction of the curve representing ascites alone from that generated in the presence of receptor yielded the expected immunoprecipitation curve, corresponding to the contribution of soluble receptor only to NGF binding (Fig. 1, lower panel). Thus both ascites fluid and hybridoma-conditioned media appeared to contain an NGF binding component that was independent of antireceptor antibody activity.

Purification of Monoclonal Antibody

In an effort to remove NGF-binding activity from the antireceptor antibody preparations, AS-ascites was applied to DEAE-cellulose in 5 mM Tris-Cl, pH 8.6, and eluted with increasing concentration of Tris-Cl. The binding and immunoprecipitation activities eluted together at approximately 0.4 M Tris-Cl, pH 8.6 (not shown). This fraction, or whole ascites, was also applied to a column (1.5 × 2 cm) of Protein A–Sepharose CL4B (Sigma) in 0.1 M sodium phosphate, pH 8.0, according to the method of Ey et al [23]. Greater than 95% of the protein and both the NGF binding and immunoprecipitating activities were recovered in the breakthrough fractions (not shown).

Gel filtration of AS-ascites on Sepharose-6B resulted in the recovery of both the NGF binding and immunoprecipitation activities in a broad peak eluting in the region of approximately 100–300 kDa. SDS-polyacrylamide gel electrophoresis under reducing conditions revealed the presence of major bands corresponding to the heavy and

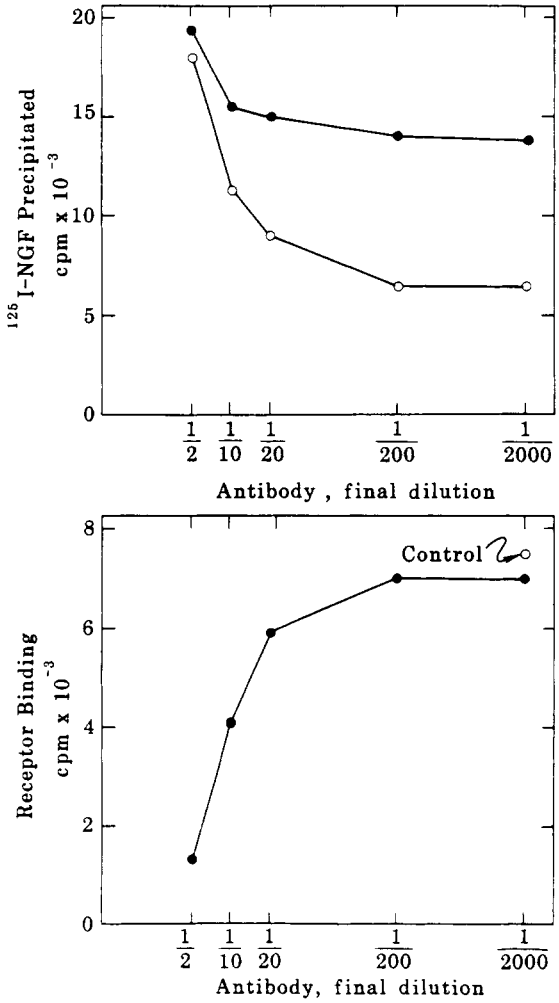

Fig. 1. Indirect immunoprecipitation of solubilized NGF receptor. Ascites (50% ammonium sulfate cut) was incubated overnight with solubilized SCG microsomes or 0.12% Triton X-100 alone and immunoprecipitated with an excess of goat antimouse immunoglobulin. The immunoprecipitation supernatants were assayed in duplicate for ^{125}I-NGF binding activity using the soluble receptor assay. Upper panel, ^{125}I-NGF binding activity in immunoprecipitation supernatants; (●), antibody + soluble receptor, (○), antibody + 0.12% Triton X-100. ^{125}I-NGF binding in the absence of antibody was 13,300 cpm in the presence of soluble receptor and 5,700 cpm in the presence of detergent only. Lower panel, NGF receptor activity in immunoprecipitation supernatants obtained by subtracting binding activity due to ascites alone from that due to ascites plus solubilized microsomes. Control value represents specific ^{125}I-NGF binding to untreated soluble microsomes obtained by subtraction of the value obtained in detergent only from that obtained with soluble receptor.

light chains of IgG (Fig. 2, inset, arrows). Application of this partially purified preparation to a column of NGF covalently linked to Sepharose 6B did not result in the specific adsorption of the NGF binding activity from the mixture.

IgG from ascites fluid was obtained in essentially pure form in the pH 6.3 breakthrough from a column of DEAE-cellulose (Fig. 3). Approximately 2 mg of

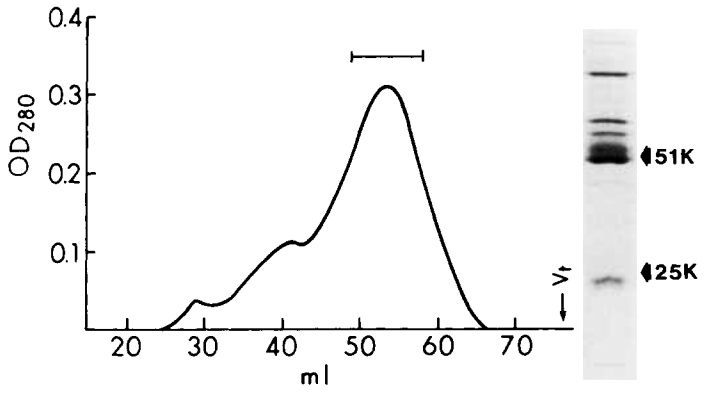

Fig. 2. Partial purification of antibody on Sepharose 6B. Ascites (1 ml) was precipitated with 50% ammonium sulfate, redissolved in H_2O, applied to a column (1.5 × 43 cm) of Sepharose 6B, and eluted with HBSS/10 mM Hepes, pH 7.5. Pooled fractions were dialyzed against H_2O and lyophilized. The partially purified material was analyzed by SDS-electrophoresis on a 10% polyacrylamide gel; Coomassie staining revealed two major bands at M_r 51,000 and 25,000 (arrows), corresponding to the heavy and light chains of IgG.

IgG was recovered from 1 ml of ascites fluid, half of which probably consists of normal serum IgG. This material was greater than 85% pure as judged by Coomassie staining (Fig. 3, inset), contained no NGF binding activity in the indirect immunoprecipitation assay, and immunoprecipitated 80% of solubilized receptor activity in the indirect assay.

Effect of Antibody on Binding of ^{125}I-NGF to Receptor

In order to determine whether of not the receptor determinant recognized by the antibody was at or near the NGF binding site, the binding of 0.2 nM ^{125}I-NGF was measured in the presence or absence of 10 μl/ml native NGF at 24°C to crude microsomes, dissociated chick embryo dorsal root ganglia, and PC12 cells. Over a 1,000-fold dilution of antibody, ^{125}I-NGF binding to each tissue was 100 ± 10% of that observed in the absence of antibody whether antibody was present in a preincubation step or added simultaneously with ^{125}I-NGF. Similarly, the binding of ^{125}I-antibody to SCG microsomes appeared to be unaffected by the presence of excess NGF (not shown).

Direct Immunoprecipitation of Solubilized NGF Receptor

Because antibody appeared to bind to receptor at a site other than the NGF binding site, it was possible to demonstrate immunoprecipitation of the NGF receptor using a direct assay. Soluble receptor was preincubated with ^{125}I-NGF in the presence or absence of 1 μM unlabeled NGF. DEAE-purified antibody was then added in increasing dilutions and the mixture was incubated overnight at 4°C. Antigen-antibody complexes were precipitated by the addition of a slight excess of goat antimouse immunoglobulin. The resulting immunoprecipitation curve (Fig. 4) revealed that the amount of precipitated radioactivity increased as the antibody concentration increased, demonstrating that the loss of PEG-precipitable ^{125}I-NGF binding activity that was observed in the indirect assay was due to antibody-mediated accumulation of receptor in the immunoprecipitate.

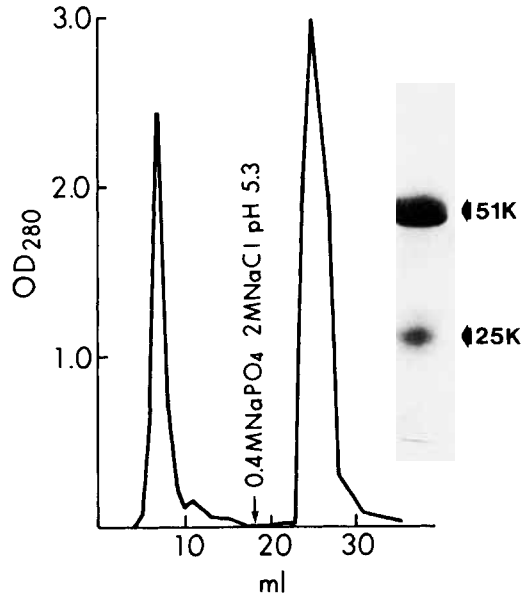

Fig. 3. Purification of antibody on DEAE-cellulose. Ascites (1 ml) was diluted with an equal volume of 35 mM sodium phosphate, adjusted to pH 6.3, and applied to a column (1.5 × 8 cm) of DEAE-cellulose equilibrated with 17.5 mM sodium phosphate, pH 6.3. IgG was collected in the breakthrough fractions, pooled, and dialyzed against HBSS/10 mM Hepes, pH 7.5. SDS-electrophoresis on a 10% polyacrylamide gel revealed only two major Coomassie-staining bands at M_r 51,000 and 25,000, corresponding to the heavy and light chains of IgG.

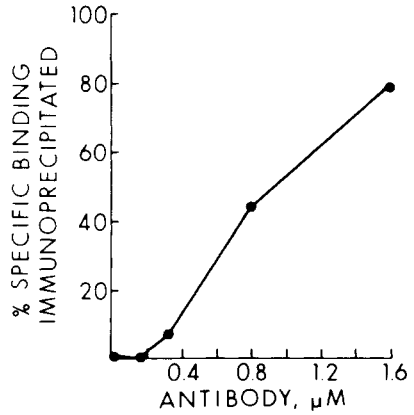

Fig. 4. Direct immunoprecipitation of solubilized NGF receptor. Solubilized microsomes were incubated in duplicate with ^{125}I-NGF in the presence or absence of unlabeled NGF for 2 hr at 24°C. Purified antibody was added and the samples incubated for 16 hr at 4°C. Immunoprecipitation was effected by addition of a slight excess of goat antimouse immunoglobulin, incubation for 4 hr at 4°C, and centrifugation in a Beckman microfuge. Specific binding was determined at each antibody concentration.

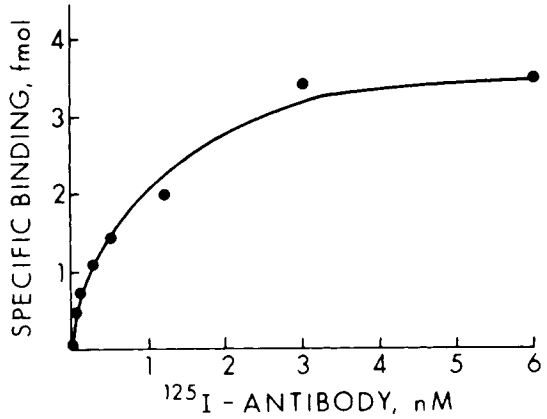

Fig. 5. Binding of ^{125}I-antibody in presence or absence of 0.3 μM unlabeled antibody for 1.5 hr at 24°C. Duplicate samples were washed and counted as described in Materials and Methods. Specific binding was determined at each point.

Binding of ^{125}I-Monoclonal Antibody to Sympathetic Membranes

Purified monoclonal antibody was iodinated to a specific activity of 20,000 dpm/fmol. Specific binding of this preparation to SCG microsomes was measurable at .05 nM ^{125}I-antibody and reached saturation by 3 nM ^{125}I-antibody (Fig. 5). Antibody bound per mg of membrane was 0.6 fmol. This value closely approximates maximal ^{125}I-NGF binding measured in this tissue (0.2–0.3 fmol/μg membranes ([13]; C.J. Morgan, unpublished results).

DISCUSSION

A major advantage of the hybridoma technique over conventional methods of obtaining antibodies is that a monospecific antiserum can be produced against an antigen that may represent a very small fraction of the immunogen. The specificity of the antibody obtained is limited by the specificity of the assay used to screen the hybridoma repertoire. In the selection of an antireceptor antibody, two types of screening procedures are commonly available: competition for ligand binding and, when a soluble receptor assay is available, antibody-mediated precipitation of receptor. The indirect immunoprecipitation assay rather than ligand binding competition was chosen to screen for antibody against the NGF receptor for two reasons. First, the immunoprecipitation assay, unlike the binding competition assay, allows detection of antibody directed against receptor determinants located at domains other than the NGF binding site. Second, by using an indirect assay, in which residual NGF binding activity is measured in the postimmunoprecipitation supernatant, antibody directed against determinants involved in the ligand binding site could be detected as well.

A variety of empirical evidence has indicated that one or more serum components may inhibit the binding of ^{125}I-NGF to its receptor, which would thus interfere with detection of an antireceptor antibody by ligand binding competition. In this study the apparent maximum immunoprecipitating activity measured in hybridoma-conditioned media was limited to 20% of total receptor in the incubation and this was

explained by the presence in media, and in much greater quantity in ascites, of an NGF binding activity precipitable by polyethylene glycol in the soluble receptor assay. The ability of 10% PEG 6000 to separate bound from free ^{125}I-NGF is dependent essentially on molecular size. Therefore, if the media NGF binding activity had been a low-molecular-weight species, it would not have been detected in the assay.

The serum component possessing NGF-binding activity has been reported to be α_2-macroglobulin [24]. Based on its elution behavior on Sepharose 6B the activity encountered here is clearly not α_2-macroglobulin. Native α_2-macroblobulin, with a molecular weight of approximately 10^6, would be expected to elute near the void volume of a Sepharose 6B column, whereas the NGF binding activity identified here coelutes with IgG in the 100-300 kDa range. That the activity is not IgG is indicated by its retention on DEAE at pH 6.3, conditions under which the antibody is eluted from the column. The binding of ^{125}I-NGF by this entity was competed for by unlabeled NGF (Fig. 1; C.J. Morgan, unpublished observations), but could not be adsorbed onto an NGF affinity column, presumably due to unfavorable conformational constraints imposed by the solid matrix to which NGF was covalently attached.

Based on its native and reduced molecular weights, the antibody produced by hybrid clone G91E4 was identified as an IgG. The antibody bound to both soluble and particulate sympathetic ganglia plasma membrane-enriched microsomes and was capable of immunoprecipitating solubilized NGF receptor in the presence of a secondary antibody whether or not the receptor was bound to NGF. Purified antibody did not affect the binding of ^{125}I-NGF to rabbit sympathetic membranes, chick dorsal root neurons, or PC12 cells, regardless of whether the antibody was present in a preincubation or added simultaneously with ^{125}I-NGF. The antibody was therefore directed against a receptor determinant not in the immediate molecular vicinity of the NGF binding site.

The antibody was radioiodinated using the lactoperoxidase method. The stability of the radioiodinated antibody was highly variable, a problem that has been encountered with other monoclonal antibodies [25] (M. Derby, personal communication). The binding of radiolabeled antibody to sympathetic membranes was of high affinity, with an estimated K_D of 0.7 nM (Fig. 5). The amount of antibody bound to membranes at saturation was very similar to the maximal amount of NGF binding of this tissue, suggesting that the number of antigenic sites approximated the number of NGF receptor sites in sympathetic membranes. This is central to a consideration of the antigenic specificity of the monoclonal antibody. Although the assay used to screen hybridoma supernatants was specific in its ability to distinguish antibody directed against a minor component (NGF receptor) of a mixture of solubilized protein, it could not be assumed that the antibody selected was directed exclusively against the antigen of choice. The presence of a limited number of membrane sites to which ^{125}I-antibody bound specifically suggested that it was indeed directed against a determinant located exclusively on the NGF receptor.

Some monoclonal antibodies directed against growth factor receptors have been found to be either agonists [25] or antagonists [26] of growth factor activity. Although the monoclonal antibody we have obtained does not affect the binding of ^{125}I-NGF to receptor, highly purified antibody preparations have been found to mimic the induction by NGF of neurite outgrowth from chick dorsal root ganglia in vitro (M. Taniuchi and E.M. Johnson, personal communication). This observation suggests that the NGF

receptor is immunologically similar across species and tissue barriers and that the receptor itself may be capable of producing the biological responses heretofore attributed to NGF. Use of the antireceptor antibody in these and other studies should help to define the structure and physiology of the NGF receptor and to clarify the role of nerve growth factor itself in the mechanism of NGF action.

ACKNOWLEDGMENTS

We thank Drs. Pamela Manning and Eugene Johnson for helpful discussion and for reviewing the manuscript, and Marian Riley for excellent technical assistance. This study was supported by U.S.P.H.S. research grant NS10229.

REFERENCES

1. Bradshaw RA, Costrini NV, Morgan CJ, Tait JF, Weinman SA: In Haber B, Perez-Polo Jr, Coulter JD (eds): "Proteins in the Nervous System: Structure and Function." New York: Alan R. Liss, 1982, pp 271–283.
2. Massague J, Guillette BJ, Czech MP, Morgan CJ, Bradshaw RA: J Biol Chem 256:9419–9424, 1981.
3. Morgan CJ: Doctoral Thesis, Washington University, St. Louis, Missouri, 1982.
4. Massague J, Buxser S, Johnson G, Czech MP: Biochim Biophys Acta 693:205–212, 1982.
5. Costrini NV, Kogan M, Kukreja K, Bradshaw RA: J Biol Chem 254:11242–11246, 1979.
6. Fernandez-Pol JA, Klos DJ, Hamilton PD: Biochem Int 5:213–217, 1982.
7. Puma P, Buxser SE, Watson L, Kelleher DJ, Johnson GL: J Biol Chem 258:3370–3376, 1983.
8. Grob PM, Berlot CH, Bothwell MA: Proc Natl Acad Sci USA 80:6819–6823, 1983.
9. Bocchini V, Angeletti PU: Proc Natl Acad Sci USA 64:787–794, 1969.
10. Bolton AE, Hunter WM: Biochem J 133:529–539, 1973.
11. Banerjee SP, Cuatrecasas P, Snyder SH: J Biol Chem 251:5680–5685, 1976.
12. Tait JF, Weinman SW, Bradshaw RA: J Biol Chem 256:11086–11092, 1981.
13. Banerjee SP, Snyder SH, Cuatrecasas P, Greene LA: Proc Natl Acad Sci USA 70:2519–2523, 1973.
14. Costrini NV, Bradshaw RA: Proc Natl Acad Sci USA 76:3242–3245, 1979.
15. Lowry OH, Rosebrough NJ, Farr AL, Randall RJ: J Biol Chem 193:265–275, 1951.
16. Markwell MA, Haas SM, Bieber LL, Tolbert NE: Anal Biochem 87:206–210, 1978.
17. Laemmli UK: Nature 227:680–685, 1970.
18. Kohler G, Howe SC, Milstein C: Eur J Immunol 6:292–295, 1976.
19. Horibata K, Harris AW: Exp Cell Res 60:61–77, 1970.
20. Galfre G, Howe SC, Milstein C, Butcher GW, Howard JC: Nature 266:550–552, 1977.
21. Littlefield JW: Science 145:709–710, 1964.
22. Levy HB, Sober HA: Proc Soc Exp Biol Med 103:250–252, 1960.
23. Ey PL, Prowse SJ, Jenkin CR: Immunochemistry 15:429–436, 1978.
24. Ronne H, Anundi H, Rask L, Peterson PA: Biochem Biophys Res Commun 87:330–336, 1979.
25. Schreiber AB, Libermann TA, Lax I, Yarden Y, Schlessinger J: J Biol Chem 258:846–853, 1983.
26. Roth RA, Cassell DJ, Wong KY, Maddux BA, Goldfine ID: Proc Natl Acad Sci USA 79:7312–7316, 1982.