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Biochemistry

Characterization of nerve growth factor receptor in neural crest tumors using monoclonal antibodies

(melanoma/membrane receptor/tumor antigen)

ALONZO H. ROSS*, PETER GROB†, MARK BOTHWELL†, DAVID E. ELDER‡, CAROLYN S. ERNST‡, NADIA MARANO†, BARBARA F. D. GHRIST*, CATHERINE C. SLEMP†, MEENHARD HERLYN*, BARBARA ATKINSON‡, AND HILARY KOPROWSKI*

*The Wistar Institute of Anatomy and Biology, 36th Street at Spruce, Philadelphia, PA 19104; †Princeton University, Princeton, NJ 08544; and ‡Department of Pathology and Laboratory Medicine, Hospital of the University of Pennsylvania, Philadelphia, PA 19104

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ABSTRACT **The nerve growth factor (NGF) receptor was characterized by using a new series of anti-receptor monoclonal antibodies (MAbs). These MAbs (*i*) showed significantly greater reactivity with a melanoma cell line expressing higher levels of NGF receptor, (*ii*) inhibited the binding of ^{125}I-labeled NGF to its receptor, and (*iii*) immunoprecipitated both metabolically labeled and ^{125}I-labeled NGF affinity-labeled receptor. These experiments defined the receptor as a 75-kDa cell-surface protein. The NGF receptor was visualized by immunoperoxidase staining in tissue sections of human nevi, melanomas, neurofibromas, a pheochromocytoma, and peripheral nerves. Uniform staining of the cytoplasm suggests that, in addition to cell-surface NGF receptors, there is a population of intracellular receptors.**

Nerve growth factor (NGF) has been the subject of extensive study because of its importance for regulation of development of sympathetic and sensory neurons and possibly other neural crest-derived cell types as well (1). Involvement of NGF in neural crest tumors such as melanoma has been suggested but never directly demonstrated. Efforts to identify oncogenes or oncogene-encoded products in malignant melanoma have been inconclusive (2), but the NGF receptor is a reasonable candidate particularly since, as we demonstrate in this study, it is expressed on melanoma cells in much greater quantities than on normal melanocytes. Progress in determining the relevance of this receptor to the transformed phenotype has been slow because the NGF receptor is not well characterized, nor are there good anti-receptor monoclonal antibodies (MAbs) or polyclonal antibodies available. From a collection of MAbs to melanoma cell surface antigens, we have identified precipitating MAbs to the NGF receptor and, using these MAbs, have carefully defined the biochemical nature of the NGF receptor and its distribution among normal and transformed neural crest tissues.

MATERIALS AND METHODS

Human Cell Lines. Human melanoma cell line WM245 was derived as described (3). Human melanoma line A875 (4) was a gift of G. J. Todaro and human melanoma line SK MEL 37, a gift of K. O. Lloyd of the Sloan–Kettering Institute. Cells were grown in minimum Eagle's medium or L15 medium supplemented with 10% fetal bovine serum.

Mouse MAbs. The preparation and characterization of anti-melanoma MAbs used in this study have been described (5) except for ME20.4 (IgG), which was prepared by using WM245 melanoma cells as immunogen in mice. MAbs were used as hybridoma culture supernatant; in some experiments, immunoglobulin from ascites prepared in pristane-primed BALB/c mice was purified by precipitation with 50% ammonium sulfate and chromatography with DEAE Affi-Gel Blue column (Bio-Rad).

Binding Assays. NGF was isolated and iodinated using lactoperoxidase as described (6, 7). For the ^{125}I-labeled NGF (^{125}I-NGF) binding assay, A875 cells were suspended in Dulbecco's phosphate-buffered saline (P_i/NaCl) with 1 mg of bovine serum albumin per ml and increasing amounts of ^{125}I-NGF (35 cpm/pg). After 1 hr at 23°C, 0.1 ml of the cell suspension was layered on top of 0.25 ml of P_i/NaCl containing 10% sucrose. After the cells were pelleted, the tube was frozen in a dry-ice slurry. The tip of the tube containing the cell pellet was cut off and assayed for radioactivity. Nonspecific binding as measured in parallel tubes containing NGF at 15 μg/ml was less than 5% of the specific binding.

Purified IgG was iodinated with lactoperoxidase (7.7 cpm/pg), and binding to cells was measured by the same procedure as for ^{125}I-NGF. Identical results were obtained whether the binding was titrated with increasing amounts of ^{125}I-labeled IgG (^{125}I-IgG) or nonradioactive IgG. Nonspecific binding of IgG under these conditions was negligible. Binding of nonradioactive IgG was measured by an indirect RIA utilizing ^{125}I-labeled rabbit anti-mouse F(ab')$_2$ immunoglobulin (Cappel Laboratories, Cochranville, PA) as described (8).

Cytofluorography. Cells to be analyzed were released from the substratum with Versene, washed once with Dulbecco's P_i/NaCl supplemented with 2% gamma globulin-free horse serum and 0.02% NaN_3 but lacking divalent cations. The cells (3.5×10^5 per sample) were resuspended in 0.1 ml of hybridoma culture supernatant for 1 hr on ice, and the volume was brought to 2 ml by the addition of P_i/NaCl containing horse serum. The cells were pelleted, resuspended in 0.1 ml of fluorescein-conjugated rabbit anti-mouse immunoglobulin at 60 μg/ml, and incubated for 1 hr on ice. The cells were washed in the same manner, resuspended in 1 ml of P_i/NaCl containing horse serum, and analyzed in an Ortho Cytofluorograf 50HH with a linear fluorescence output divided into 200 channels. Generally, 3000 cells were counted per assay.

Affinity-Labeling, Immunoprecipitation, and Electrophoretic Analysis of the NGF Receptor. The procedures for affinity-labeling, immunoprecipitation, and NaDodSO$_4$/polyacrylamide gel electrophoresis have been described (9, 10).

Immunoperoxidase Staining of Frozen and Fixed Tissue. Tissues obtained fresh from nevi and melanomas of patients seen by the Pigmented Lesion Group (Hospital of the Uni-

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Abbreviations: NGF, nerve growth factor; MAb, monoclonal antibody.

versity of Pennsylvania) were sampled, and representative portions were stored at −70°C. Cryostat sections (5 μm) were cut, dried overnight, fixed in acetone for 10 min, and allowed to dry. After a 3-min wash in P_i/NaCl, the sections were successfully incubated for 15 min with hybridoma culture supernatant (1/50), biotinylated anti-mouse antibody (1/75) (Vector Laboratories, Burlingame, CA), and avidin–biotinylated peroxidase complex (Vector Laboratories) with 3-min washes in P_i/NaCl between all steps. After another P_i/NaCl wash, aminoethylcarbazole (20 mg of aminoethylcarbazole in 5 ml of *N,N*-dimethylformamide mixed with 95 ml of 0.05 M acetate buffer, pH 5.2/0.03% H_2O_2) was applied for 10 min. Sections were rinsed in water, counterstained with hematoxylin, dehydrated, and mounted.

Tissue from the files of the Pigmented Lesion Group or the Division of Surgical Pathology (Hospital of the University of Pennsylvania) and a cell pellet of A875 cells were fixed in Bouin's solution or neutral buffered formalin and were paraffin-embedded. Sections (5 μm) were deparaffinized, washed for 5 min in running water, and stained as above.

RESULTS

Correlation Between Expression of Melanoma-Associated Antigens and NGF Receptors. To identify MAbs that might be directed against the NGF receptor, the reactivities in RIA of eight anti-melanoma MAbs with A875, a human melanoma line unusually rich in NGF receptor (4), and SK MEL 37, a human melanoma line with far fewer NGF receptors, were compared (Table 1). Only ME82-11 and ME20.4 preferentially bound to A875 cells. Flow cytofluorometric analysis of A875 cells labeled with murine MAbs and a fluorescein-conjugated second antibody (Fig. 1) indicated that 97% of the A875 cells were positive for MAbs ME82-11 and ME20.4, and, in agreement with the RIA results, the quantity of IgG bound was much greater than that for two other anti-melanoma MAbs, ME31.3 and ME37-7.

The correlation between MAb and NGF binding was quantitated with A875 cells. Scatchard analysis (11) of the binding of ME20.4 ^{125}I-IgG demonstrated 0.8×10^6 binding sites per cell with a dissociation constant of 7×10^{-10} M (Fig. 2). Simultaneous measurements of ^{125}I-NGF binding to A875 cells gave 1×10^6 sites per cell with a dissociation constant of 2×10^{-9} M.

Immunochemical Identification of the ME20.4 Antigens. We then tested the MAbs for their ability to inhibit binding of ^{125}I-NGF. Hybridoma culture supernatants of ME82-11 or ME20.4 but not P3X63Ag8, a nonspecific MAb control, strongly inhibited binding (Fig. 3*A*). Culture supernatants

Table 1. Expression of melanoma-associated antigens and NGF receptors by human melanoma cell lines

Ligand	Bound cpm* to SK MEL 37	Bound cpm* to A875	Receptor
NGF	444†	8,371†	NGF receptor
ME77-71	1,950	0	None identified
ME20.11	3,224	0	Protein (28)‡
ME31.3	2,932	804	Chondroitin sulfate proteoglycan
ME82-11	912	11,592	Protein (75)‡
ME20.4	1,288	12,872	Protein (75)‡
ME312	1,626	0	Ganglioside
ME061	9,837	1,210	Protein (97)‡
ME37-7	17,427	11,267	HLA-DR

*Determined by indirect RIA. The nonspecific binding (950 cpm) with P3X63Ag8 was subtracted.
†Determined by direct binding of ^{125}I-NGF.
‡The number in parentheses is the $M_r \times 10^{-3}$, as determined by NaDodSO$_4$/polyacrylamide gel electrophoresis (5).

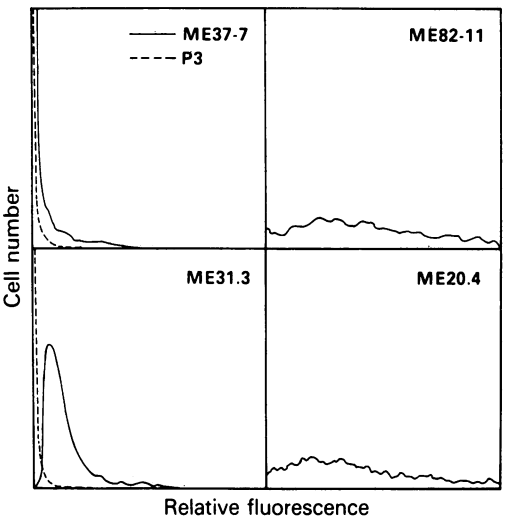

FIG. 1. Cytofluorographic analysis of antigen expression on human melanoma cells. A875 cells were labeled with murine MAbs and a fluorescein-conjugated second antibody as described. The antigens specified by MAbs ME37-7 and ME31.3 are HLA-DR and the melanoma-associated proteoglycan antigen, respectively. ME82-11 and ME20.4 are the putative anti-NGF receptor MAbs. The nonspecific control MAb P3X63Ag8, which contains MAb MOPC 21, is shown (*Left*) as a dashed line.

from hybridomas ME83-29, ME82-13, ME80-25, and ME81-22, all of which resulted from the same fusion as ME82-11, had similar specificities, and may synthesize the same MAb, also strongly inhibited binding of ^{125}I-NGF. Binding was inhibited in a dose-dependent manner by purified MAbs (Fig. 3*B*), requiring 700 ng of ME82-11 IgG and 300 ng of ME20.4 per ml for 50% inhibition.

The direct interaction between the MAbs and the NGF receptor was demonstrated by using affinity-labeled NGF receptor. A875 cells were incubated with ^{125}I-NGF and with the crosslinking agent ethyldimethylisopropylaminocarbodiimide and then were solubilized with detergent. The apparent molecular weight of the affinity-labeled NGF receptor in this gel system was 90,000, which is slightly lower than previously reported (12). A second, much weaker band of apparent M_r 200,000 was detected in some but not all experiments. The affinity-labeled NGF receptor was immunoprecipitated with MAbs ME20.4, ME81-22, ME80-25, ME82-13,

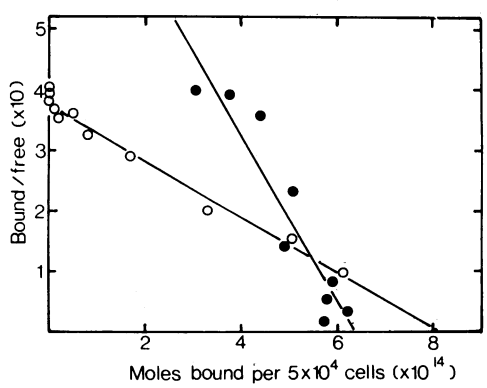

FIG. 2. Scatchard plot of binding of ^{125}I-NGF and ME20.4 ^{125}I-IgG to A875 cells. A875 cells (5×10^5 cells per ml in P_i/NaCl supplemented with bovine serum albumin at 1 mg/ml) were incubated for 1 hr at 23°C with various concentrations of ^{125}I-NGF or with 150 ng of ME20.4 ^{125}I-IgG per ml with various concentrations of unlabeled ME20.4 IgG. Aliquots (0.1 ml) were removed, the cells were sedimented, and bound ligand was determined as described. The data were fitted by the least-squares method. ○, NGF binding; ●, ME20.4 IgG binding data.

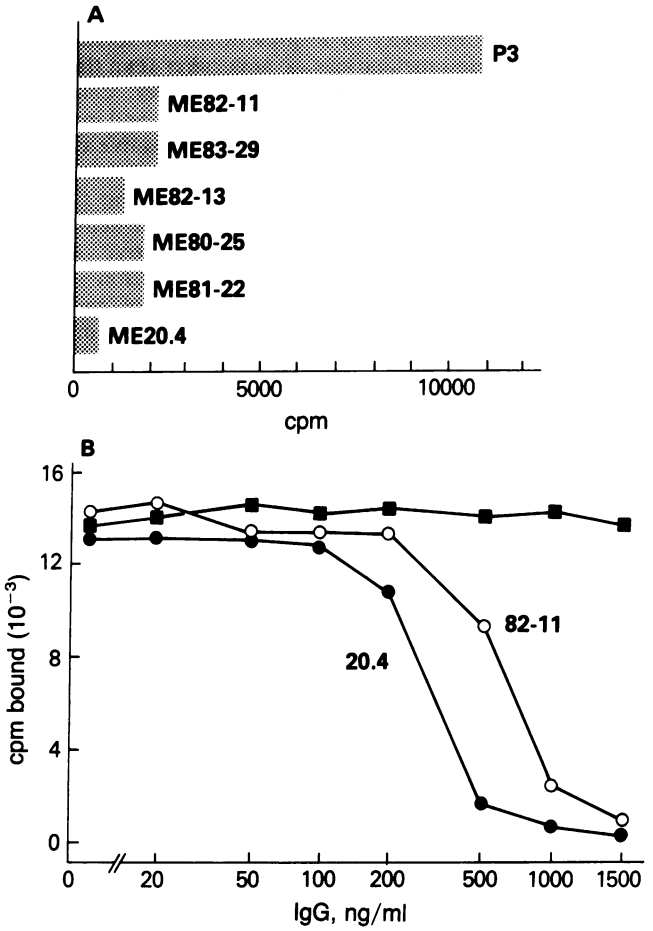

FIG. 3. Inhibition of ^{125}I-NGF binding to A875 cells by anti-NGF receptor MAbs. ^{125}I-NGF (100,000 cpm) was added to human melanoma cells (4×10^5) preincubated for 30 min at 37°C with murine immunoglobulins. After an additional 30-min incubation at 37°C, the cells were pelleted through 10% sucrose and assayed for radioactivity in a gamma counter. Nonsaturable binding, measured in the presence of 10 μg of unlabeled NGF per ml, was subtracted. (*A*) Inhibition by hybridoma culture supernatants. P3 is the nonspecific culture supernatant P3X63Ag8. The other culture supernatants contain the putative anti-NGF receptor MAbs. (*B*) Inhibition by increasing concentrations of purified immunoglobulins. ●, ME20.4; ○, ME82-11; ■, normal mouse IgG.

ME83-29, and ME82-11 (Fig. 4, lanes 2–7) but not with P3X63Ag8 or P_i/NaCl (Fig. 4, lanes 8 and 9). When detected, the M_r 200,000 band was also immunoprecipitable with ME20.4. Thus, we conclude that these MAbs are directed against the cell surface NGF receptor.

The molecular weight of the receptor should differ from that of the crosslinked receptor by the molecular weight of one NGF monomer (M_r 13,000). This prediction was verified by immunoprecipitation of the receptor from detergent extracts of cells metabolically labeled with [^{35}S]cysteine (Fig. 5). The immunoprecipitated receptor resolved in electrophoresis as a slightly broadened band with an apparent M_r of 75,000. The broadening is due to heterogeneous glycosylation of the receptor (unpublished data).

Application of MAb ME20.4 as a Histochemical Reagent. MAb ME20.4 was used to stain both frozen and fixed tissue sections in an effort to better define the distribution of the NGF receptor (Fig. 6 and Table 2). There was no evident staining of normal melanocytes. This assignment could be made since, in the original color image of these sections, the brown-colored melanin and the red immunoperoxidase dye were easily distinguished. Staining of primary and metastatic

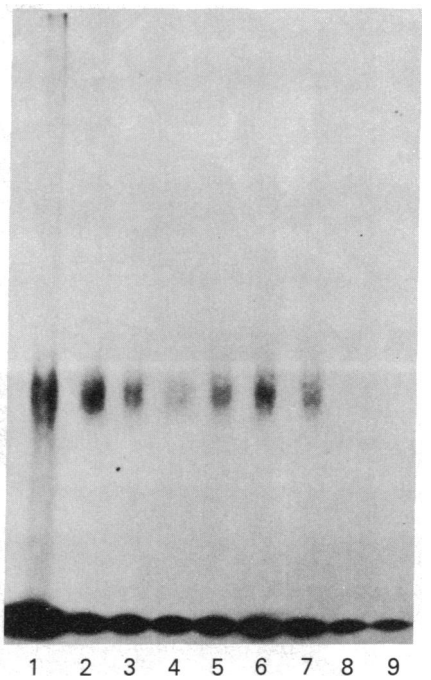

FIG. 4. Immunoprecipitation of NGF receptor affinity-labeled with ^{125}I-NGF. A875 cells (1×10^6) in suspension were incubated with ^{125}I-NGF (20 ng/ml) for 30 min at 37°C, treated with 4 mM ethyldimethylisopropylaminocarbodiimide for 15 min at 22°C, extracted with detergent, and clarified by ultracentrifugation. The supernatant was either analyzed directly by NaDodSO$_4$ gel electrophoresis (lane 1) or immunoprecipitated with murine MAbs (lanes 2–8). An autoradiogram of the dried gel is shown. Lanes: 1, extract of affinity-labeled A875 cells; 2, immunoprecipitation after incubation with ME20.4; 3, with ME81-22; 4, with ME80-25; 5, with ME82-13; 6, with ME83-29; 7, with ME82-11; 8, with nonspecific control P3X63Ag8; 9, with P_i/NaCl.

melanomas, neurofibromas, pheochromocytoma, and dysplastic or common nevi was diffuse throughout the cytoplasm and/or cell membranes, suggesting that the receptor

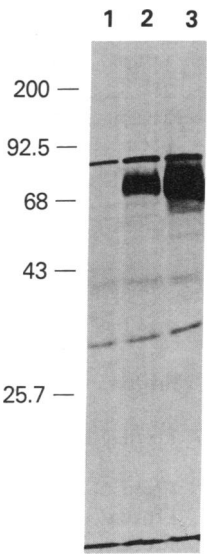

FIG. 5. Immunoprecipitation of metabolically labeled NGF receptor. A875 cells were labeled with [^{35}S]cysteine and extracted with detergent. After clarification by ultracentrifugation, the NGF receptor was immunoprecipitated with murine MAbs and analyzed by NaDodSO$_4$ gel electrophoresis and fluorography. Lanes: 1, nonspecific control MAb P3X63Ag8; 2, ME82-11; 3, ME20.4. Numbers indicate $M_r \times 10^{-3}$.

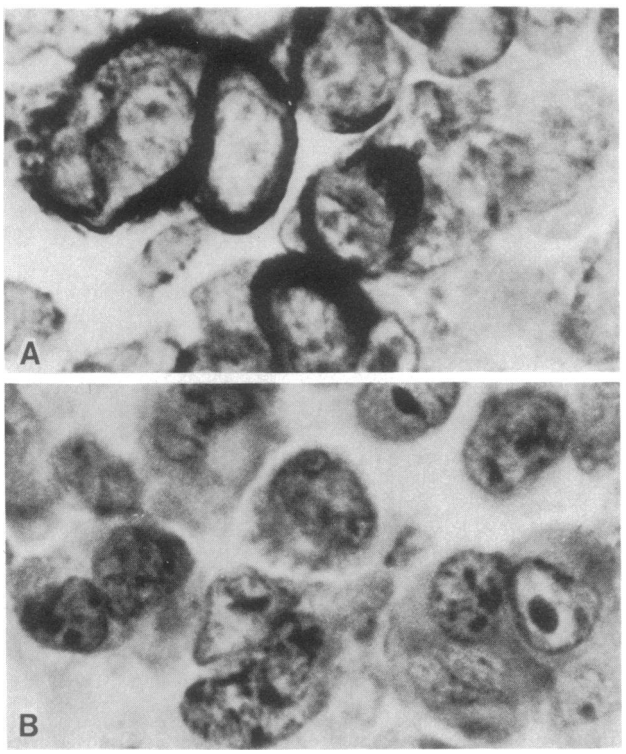

FIG. 6. Detection of NGF receptor by immunoperoxidase staining. Fixed A875 cells were stained with either ME20.4 (*A*) or P3X63Ag8 (*B*). Note the strong but heterogeneous peripheral staining. (×3600.)

may be cytoplasmic as well as plasma membrane-associated. The staining of A875 cells, on the other hand, was strongly localized at the cell periphery, suggesting that the bulk of the receptor in this cell line is restricted to the plasma membrane. Staining of melanoma and nevus cells was typically heterogeneous, ranging from 10% to 50% of the cells in melanoma sections. One dysplastic nevus showed 100% cellular reactivity, whereas two other nevi showed 10% reactivity. In contrast to the reactivity of lesions in frozen sections, no paraffin-embedded melanomas or nevi other than the A875 cell line were positive for staining. However, cutaneous nerves reacted in five of five paraffin samples and in all frozen sections tested, serving as an internal positive control. This staining was localized in the perineurium and endoneurium and/or Schwann cell cytoplasm, indicating that the neural sheath is rich in NGF receptor. Of four neurofibromas examined as fixed sections, two were positive. A single pheochromocytoma examined in frozen section showed strong cytoplasmic and/or membrane staining of small, presumably neoplastic cells. Only a few of the larger tumor cells stained. Sections of pancreas, adrenal, and salivary glands, obtained at autopsy, showed positive staining of 5% of submandibular-gland acinic cells in frozen sections. Control experiments with nonspecific MAb P3X63Ag8 gave no staining with any of the samples.

Table 2. Summary of immunoperoxidase staining with ME20.4 of frozen and fixed tissue sections

Tissue	Fixation	Ratio of positive/total*	Site of staining
Melanocytes	Frozen	0/11	—
	Fixed	0/5	—
Nevi	Frozen	5/5	Cytoplasmic
	Fixed	0/3	—
Melanomas	Frozen	6/9	Cytoplasmic
	Fixed	0/3	—
A875 melanoma cell line	Frozen	1/1	Plasma membrane
	Fixed	1/1	Plasma membrane
Peripheral nerves	Frozen	8/8	Neural sheath
	Fixed	5/5	Neural sheath
Neurofibromas	Fixed	2/4	Cytoplasmic
Pheochromocytomas	Frozen	1/1	Cytoplasmic
Pancreas†	Frozen	0/1	—
Adrenal gland	Fixed	0/1	—
Submandibular gland	Frozen	1/1‡	Cytoplasmic
	Fixed	1/1§	Cytoplasmic

*Ratio of positive samples to the total number of samples examined.
†Autopsy tissue, 5-hr postmortem.
‡Staining of 5% of acinic cells.
§Staining of scattered ductular basal cells.

DISCUSSION

The experiments described here demonstrate that six of our anti-melanoma MAbs from two independent fusions are directed against the NGF receptor. The MAbs all have similar properties and may be directed against epitopes proximal to the NGF binding site since they all inhibit NGF binding and compete with each other in binding to the cell surface (unpublished). In fact, five of the MAbs that resulted from a single fusion may be identical. The MAbs are specific for the human NGF receptor—we have not been able to demonstrate any reactivity with rat pheochromocytoma PC12 cells or chick embryonal sensory neurons (not shown). Other investigators have described MAbs that interact with the NGF receptor (13), but these MAbs do not immunoprecipitate the NGF receptor. Thus, the MAbs described here are uniquely useful for the biochemical characterization of the receptor.

Electrophoretic analysis of immunoprecipitates reveals a M_r 75,000 receptor protein. A M_r 200,000 species is also observed when 2-mercaptoethanol is omitted from $NaDodSO_4$ sample buffer (unpublished data), suggesting that the M_r 200,000 protein may contain the M_r 75,000 protein within a disulfide-stabilized oligomer. These molecular weights are in good agreement with several recent reports describing NGF receptors on rat PC12 pheochromocytoma cells and A875 human melanoma cells (9, 14, 15) in which a M_r 80,000–87,000 species and a minor M_r 200,000 receptor species were observed. We have not observed a receptor protein species corresponding to the M_r 130,000–140,000 described in rabbit sympathetic ganglia (14) or in PC12 cells (16).

Previous studies have suggested multiple forms of the NGF receptor. NGF receptors are heterogeneous with respect to kinetics and affinity of NGF binding (17, 18), but it is likely that these receptor forms are interconvertible and represent a single receptor protein (12, 16–21). The anti-receptor MAbs will provide a means of determining how the several molecular forms of the NGF receptor relate to the observed heterogeneity of binding properties.

Relatively little is known about the distribution of NGF receptors in normal and neoplastic tissue due to the previous lack of suitable specific procedures for localization *in situ*. Initial experiments have demonstrated that the anti-receptor MAbs are well suited for immunocytochemical localization of receptors, particularly on frozen sections, although specific staining of fixed tissues was also observed where NGF receptors were especially abundant. Immunoperoxidase staining has revealed NGF receptors in a variety of tissues of neural crest origin. Staining of melanomas, nevi, and neurofibromas was diffuse throughout the cytoplasm and/or cell membranes, suggesting that the receptor may be cytoplasmic as well as plasma membrane-associated. An exception

was melanoma cell line A875, in which the staining was mostly confined to the plasma membrane. Staining of peripheral nerves was confined to the periphery, perhaps involving sheath cells, which are also of neural crest origin (22). This assignment would be consistent with the reported presence of NGF receptors on glial cells of sensory ganglia (23) and the suggestion (24) that NGF might act directly on nonneuronal cells in its target tissue with either secondary or concomitant effects on neurons. Any staining of axons might not have been visible due to their minute diameter. The staining of the submandibular gland, which is not known to be of neural crest origin, may be due to the unusually close association of the acinic cells with synapses from sympathetic neurons (25).

In the light of the recent discoveries linking growth factor–growth-factor receptor systems with oncogenesis (26), it is worth considering whether the abundant expression of NGF receptors on certain tumors is of consequence for their neoplastic growth. The anti-receptor MAbs have revealed that melanomas both in culture (5) and *in vivo* (Table 2) express much greater amounts of the NGF receptor than do normal melanocytes.

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