Purification of the Receptor for Nerve Growth Factor from A875 Melanoma Cells by Affinity Chromatography*

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The receptor for nerve growth factor (NGF) has been purified to near homogeneity from octylglucoside extracts of A875 melanoma cell membranes by the use of repetitive affinity chromatography on NGF-Sepharose. Elution of purified receptor (NGF receptor) was accomplished with 0.15 M NaCl, pH 11.0, containing phosphatidylcholine and octylglucoside. Chromatography on two columns of NGF-Sepharose yielded a 1500-fold purification of the receptor, as assessed by 125I-NGF binding, and permitted recovery of 9% of the total binding activity in the soluble extract. Scatchard analysis of equilibrium binding of 125I-NGF provided similar KD values for NGF receptors in soluble extracts of A875 membranes (2.2 nM) and with purified NGF receptor (3.1 nM). Examination of NGF receptor after electrophoresis on sodium dodecyl sulfate-polyacrylamide gels revealed the presence of two major peptides, of M, = 85,000 and M, = 200,000. Affinity labeling experiments, done with 125I-NGF and A875 cells, soluble extracts of A875 cell membranes, and purified receptor, show that both of these components of the NGF receptor can be specifically cross-linked to 125I-NGF.

Nerve growth factor produces a variety of diverse morphological and biochemical alterations in target tissues of neural crest origin, including effects on growth, survival, neurite outgrowth, and levels of certain enzymes (see review by Green et al., 1980). While all of these effects do not necessarily reflect those of endogenous nerve growth factor there is ample evidence that endogenous NGF plays a role in neuronal development and functioning. Numerous studies have defined the ability of NGF antibodies to interfere with development and function of neuronal tissues (Angeletti et al., 1971; Aloe and Levi-Montalcini, 1979; Gorm and Johnson, 1979, 1980). Other studies have indicated that endogenous NGF is produced by effector organs and retrogradely transported to nerve cell bodies (Hendry, 1975a,b; Hendry and Campbell, 1976; Levi-Montalcini et al., 1975).

The mode of action of NGF in initiating these effects is poorly understood at the molecular level, in part due to the fact that it has thus far been impossible to study purified NGF receptors to determine their structure and relationship to other cellular and membrane components. NGF receptors are in too low a concentration in most target tissues used for binding studies (mouse embryonic ganglia, rabbit superior cervical ganglia, PC12 pheochromocytoma cells) to permit even a partial purification of the receptor. The isolation of the human melanoma cell line A875, by Fabricant et al. (1977) provided a suitable source from which to purify NGF receptors. A875 cells possess large numbers of high affinity receptors for NGF (5 × 10^5 receptors/cell) and have been shown to respond to NGF with increased survival in serum-depleted medium. In this study, we report the purification of the nerve growth factor receptor from A875 melanoma cells.

EXPERIMENTAL PROCEDURES

Materials

Dubeczko's modified Eagle's medium and fetal calf serum were obtained from Grand Island Biological Co. Octylglucoside and N-Hydroxysuccinimidyl-4-azidobenzoate were supplied by Calbiochem-Behring. Lactoperoxidase and glucose oxidase were obtained from Sigma. N-Hydroxy succinimidyl-4-azidobenzoate was obtained from Pierce Chemical Co. Iodinations were performed with carrier-free Na-125I (Amersham Corp.) with a specific activity of 14 mCi of Na-125I/μg of iodine. All reagents for polyclarameide gel electrophoresis were obtained from Bio-Rad.

Methods

Cell Culture.—The human melanoma cell line A875 was a gift from Dr. G. Todaro (National Institutes of Health). The cells were grown in 850-ml roller bottles (Falcon) in Dubeczko's modified Eagle's medium supplemented with 10% fetal calf serum.

Preparation of A875 Membranes.—A875 cells were removed from roller bottles by scraping the sides of the bottle with a rubber policeman, and harvested in 0.05 M NaCl, 5 mM KCl, 0.9 mM CaCl2, 1.0 mM MgCl2, 10 mM HEPES, pH 7.2, 1.0 mM CaCl2. Harvesting solution and membrane isolation solutions contained the protease inhibitors phenylmethylsulfonyl fluoride (1 mM), aprotonin (20 Komberg International Units/ml), and bacitracin (100 μg/ml). Membranes were obtained from washed cells by a modification of the method of Thom et al. (1977). Membranes were stored in a phosphate-buffered saline, referred to as PBS-1 (0.137 M NaCl, 2.7 mM KCl, 0.9 mM CaCl2, 0.49 mM MgCl2, 1.47 mM KH2PO4, 0.84 mM Na2HPO4, pH 7.4) at -85 °C. Approximately 50 mg of membrane protein was obtained from 50 roller bottles.

Purification of NGF—β-NGF was purified from the submaxillary glands of adult male mice (obtained from Charles River Breeding Laboratories) by the method of Mobley et al. (1976). Before and after iodination, β-NGF migrated as a single band on 15% SDS-polyacrylamide gels and induced neurite outgrowth in PC12 cells.

Preparation of NGF-Sepharose.—Purified β-NGF was coupled to CNBr-activated Sepharose 4B (Pharmacia) for 16 h at 4 °C in 0.1 M borate, pH 8.5, 0.5% NaCl. One mg of NGF was used to prepare 1 ml of gel. After coupling was completed, the gel was blocked by incubation in 0.2 M glycine pH 8.0. The gel was washed in three alternating cycles of 0.1 M borate, pH 8.0, and 0.1 M acetic acid, pH 4.0, each containing 1 M NaCl. The gel was then resuspended in a phosphate-buffered saline solution referred to as PBS-2 (10 mM sodium phosphate, pH 7.20, 0.136 M NaCl, 0.5 mM KCl) containing 0.01% NaN3 and stored at 4 °C until use. Incorporation of NGF onto the gel was assessed by two criteria: 1) after completion of

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was permitted to proceed for 10 min. Sodium azide was added to terminate the reaction, and the membranes were washed twice in phosphate-buffered iodide (10 mM sodium phosphate, pH 7.2, 0.136 M NaCl, 5 mM KCl) by resuspension after centrifugation at 100,000 x g for 20 min. The final washed pelleted was resuspended in PBS-2.

Incorporation of label was 25%.

Affinity Chromatography of Solubilized Nerve Growth Factor Receptor—Iodination of solubilized crude and purified NGF receptor was performed using chloramine-T (Siegel et al., 1981). Two µg of protein was labeled in a mix containing 0.5 mCi of carrier-free Na125I and 0.45 mM chloramine-T for 5 min at room temperature. The sample was swirled gently during iodination. After addition of 1.5 mM sodium metabisulfite, free iodine was removed on a small column of Sephadex G-50 in PBS-2, 1% octylglucoside. Incorporation of label was 75%.

RESULTS

Affinity Chromatography of Solubilized Nerve Growth Factor Receptor—Octylglucoside extracts of A875 membranes were used to purify the NGF receptor. Triton X-100, successfully used in the past to solubilize NGF receptors (Costrini et al., 1979), yielded poor chromatographic resolution during purification of A875 NGF receptor. Octylglucoside not only permitted better resolution, but it could be easily removed prior to reconstitution of receptor into lipid vesicles. Octylglucoside at a concentration of 2%, was found to be as efficient as 1% Triton X-100 in solubilizing membranes (Table I). The apparent increase in total binding that is seen in detergent extracts is probably due to the exposure of NGF receptors from membrane vesicles. This may be due to a combination of outside-in vesicles and latent receptors exposed by detergent solubilization. Crude extracts maintained stable, reproducible specific binding for NGF for 1 week at 4°C and for several weeks at -85°C.

Initial experiments indicated that treatment with relatively small amounts of NGF-Sepharose depleted octylglucoside

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\text{Table I} \\
\begin{array}{|c|c|c|}
\hline
\text{Sample} & \text{Total binding} & \text{Specific binding} \\
& \text{pmol/mg protein} & \text{pmol/mg protein} \\
\hline
\text{A875 membranes} & 0.5 & 0.4 \\
1% Triton X-100 extracts & 2.1 & 1.7 \\
2% octylglucoside extracts & 2.5 & 1.5 \\
\hline
\end{array}
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A875 membranes were solubilized for 1 min at 0°C. After centrifugation at 100,000 x g for 30 min, 10 µl of the resultant supernatants, containing solubile extracts, were assayed for 125I-NGF binding using 0.5 µM 125I-NGF. Nonspecific binding was determined by parallel incubations in the presence of 10 µM unlabeled NGF.
extracts of binding activity in a dose-dependent manner (Table II). While a 4-fold increase in the amount of NGF-Sepharose clearly removed the NGF receptor from soluble extracts, reducing specific binding from 14.7 fmol of $^{125}$I-NGF to 3.7 fmol of $^{125}$I-NGF, glycine-treated Sepharose had no effect. Similar results were obtained when bovine serum albumin-Sepharose was used as a control (data not shown). These results clearly demonstrated that NGF-Sepharose acts as a true affinity column. Adsorption of the NGF receptor could only be accomplished with NGF, and not by a nonspecific protein (bovine serum albumin) or by the charged groups on glycine-treated Sepharose.

In subsequent experiments, when crude A875 extracts were chromatographed on NGF-Sepharose, it was found that adsorbed NGF receptor could be eluted with 0.15 M NaCl, pH 11.0, containing 1.2% octylglucoside and phosphatidylcholine. The receptor had excellent specific binding and stability when eluted by this relatively gentle procedure. Table III presents a summary of the NGF receptor purification. Receptor fractions obtained after one round of NGF-Sepharose treatment contained 0.8% of the total protein and 50% of the total binding activity added to the column. This represents a 60-fold enrichment in binding activity over the crude extract. To assess recovery of protein and monitor the proteins being purified by NGF-Sepharose chromatography, octylglucoside extracts were prepared from A875 membranes iodinated using a lactoperoxidase procedure. After the NGF receptor was purified on two sequential NGF-Sepharose columns, the fractions containing the NGF receptor binding activity contained only 0.005% of the protein originally loaded on the first column. Purified fractions, however, contained 9% of the total binding activity in the original extract. This represents a 1500-fold enrichment in binding activity in purified receptor as compared to crude extracts. Preliminary experiments involving $^{125}$I-NGF binding assays (data not shown) had indicated that the NGF receptor in our membrane preparations comprised approximately 0.1% of the total membrane protein. It was assumed, in calculating this estimate, that the receptor possessed $M_r = 100,000$ (Massague et al., 1981). On the basis of this estimate, it was predicted that a 1000-2000-fold purification would be necessary to purify the NGF receptor. Thus, the 1500-fold purified NGF receptor eluted from the second NGF-Sepharose column may be at or near homogeneity.

Use of harsher elution buffers, containing chaotropic salts or strong acids and bases, substantially reduced specific binding (data not shown). The 0.15 M NaCl elution solution, adjusted to pH 11 with 1 N NaOH, has little buffering capacity, while the high pH of this solution is adequate to break the NGF-NGF receptor interaction on the affinity column. The composition of the elution solution, however, is not sufficient to cause denaturation of the receptor binding site during brief incubations. Fractions with eluted receptor are easily adjusted to neutrality with sodium phosphate, further lessening any possibility that the receptor may be denatured during purification. Addition of phosphatidylcholine to chromatography buffers was needed to maintain stable binding activity. Initial attempts to purify the NGF receptor on DEAE-Sephasil, hydroxyapatite, and NGF-Sepharose without addition of lip-
ids to chromatography buffers gave poor recoveries of binding activities and little purification (data not shown).

**Analysis of Component Polypeptides of Crude and Purified NGF Receptors on SDS-Polyacrylamide Gels**—Fig. 1 shows autoradiographs of SDS gels containing NGF receptor purified by NGF-Sepharose chromatography of octylglucoside extracts from iodinated A875 membranes. The fraction enriched for NGF binding after one round of chromatography on NGF-Sepharose shows six polypeptides, of $M_r = 200,000$.

**Fig. 2.** SDS-polyacrylamide gel electrophoresis of A875 soluble extracts and purified NGF receptor radiiodinated with chloramine-T. Unlabeled crude extracts and purified NGF receptor were labeled with $^{125}I$ by the use of chloramine-T. Solubilized samples, each containing 45,000 cpm were electrophoresed on 7.5% separating gels. Gels were stained with Coomassie blue R-250, dried, and exposed to Kodak X-Omat R film with one intensifying screen. The autoradiographs contain soluble extract (track 1) and NGF receptor purified on two columns of NGF-Sepharose (track 2).

**Fig. 4.** $^{125}I$-NGF binding in the presence of increasing amounts of purified NGF receptors. Purified NGF receptors were assayed for $^{125}I$-NGF binding in the presence of $1 \text{ nM}^{125}I$-NGF. Nonspecific binding was determined by parallel incubations in the presence of $10 \mu g/ml$ of unlabeled NGF. NGF receptor-$^{125}I$-NGF complexes were precipitated in 20% polyethylene glycol, PBS-2 and filtered onto Amicon 0.45-µm filters, as described in the text. Each point is the result of duplicate assays. ●, total $^{125}I$-NGF bound; △, nonspecifically bound $^{125}I$-NGF; ○, specifically bound $^{125}I$-NGF.

**Fig. 3.** Affinity labeling of NGF receptors in A875 cells and of solubilized NGF receptors. Intact cells or solubilized NGF receptors reconstituted into lipid vesicles were incubated with $1 \text{ nM}^{125}I$-NGF. Nonspecific binding was determined by parallel incubations in the presence of $10 \mu g/ml$ of unlabeled NGF. NGF receptor complexes were covalently linked using N-hydroxysuccinimidy1-4-azidobenzoate, as described under "Experimental Procedures." Solubilized samples were electrophoresed on 7.5% separating SDS gels. Autoradiographs were prepared from dried Coomassie-stained gels by exposure to Kodak X-Omat film with two DuPont intensifying screens. A, tracks contain A875 cells labeled with $^{125}I$-NGF in the absence (1) or presence (2) of unlabeled NGF. Samples contained 28,000 cpm. B, tracks contain: A875 soluble extracts affinity labeled in the absence (1) and presence (2) of unlabeled NGF; NGF receptors, purified on two columns of NGF-Sepharose, affinity labeled in the absence (3) and presence (4) of unlabeled NGF; phosphatidylincholine vesicles alone affinity labeled in the absence (5) and presence (6) of unlabeled NGF. Samples each contained 13,000 cpm.
Affinity Labeling of NGF Receptor 

NGF receptor and purified receptor yielded equilibrium dissociation constants \( K_d \) of 2.2 and 3.1 nM, respectively. The concentration of NGF receptor after two passages through NGF-Sepharose was increased 3000-fold to 28,800 pmol of NGF receptor/mg of protein in the crude soluble extract. This calculation, obtained from analysis of binding done over a wide range of \( ^{125}\text{I}-\text{NGF} \) concentrations (0.1-10.0 nM), is in good agreement with the degree of purification calculated from independent experiments shown in Table III.

**DISCUSSION**

The receptor for nerve growth factor was purified from membranes of A875 melanoma cells by repetitive affinity chromatography. The purity of the receptor was ascertained by several different criteria, all of which indicate the NGF receptor in these preparations was near homogeneity. Preliminary estimates had indicated that a 1000-2000-fold purification of receptor would yield a homogeneous preparation. The increased in specific binding and receptor concentration seen in purified NGF receptors, as compared to octylglucoside extracts of A875 membranes, indicate that a 1500-3000-fold purification was accomplished. Nine per cent of the receptor from crude extracts was recovered as pure receptor, which indicates that the techniques used allow a sufficient recovery.
of material for use in further characterization of the NGF receptor.

The mild elution conditions used for NGF receptor purification appear to yield a high percentage of receptor with high specific binding and similar binding affinities to receptor in crude soluble extracts. The estimates of purification in Table III were all based on analysis of specific binding of $^{125}$I-NGF. Purified receptor maintains stable, reproducible specific binding, up to several weeks after isolation, when stored at -85 °C. The affinity chromatography procedure described in this paper can yield microgram amounts of functional NGF receptor in 2–3 h.

Examination of component polypeptides of the NGF receptor on SDS-polyacrylamide gels also indicated that NGF receptor was substantially purified after affinity chromatography. Purified receptor was markedly enriched in two peptides, of $M_{r} = 200,000$ and $M_{r} = 85,000$, when compared to soluble extracts. NGF receptors purified from lactoperoxidase-iodinated membranes and NGF receptors iodinated after purification, using chloramine-T, displayed almost identical patterns when separated on SDS-polyacrylamide gels. The shift in $M_{r}$ from 85,000 to 80,000 seen in the lower molecular weight peptide of the NGF receptor after chloramine-T treatment may well be due to some damage inflicted on receptor proteins by the harsher chloramine-T oxidation. We have preliminary evidence that the NGF receptor is a glycoprotein, based on its binding to wheat germ agglutinin-Sepharose. Oxidation by chloramine-T may have caused removal of carbohydrate moieties from the NGF receptor and altered its mobility in this manner. The broad pattern seen with the $M_{r} = 85,000$ band of the NGF receptor extending the band over a range of $M_{r}$ values, from 93,000 to 70,000, has been seen with other membrane glycoproteins. The $M_{r} = 85,000$ component of the NGF receptor does appear to be more labile to alteration than the $M_{r} = 200,000$ band to prolonged storage. Its $M_{r}$ shifts to even lower values of approximately 80,000.

The $M_{r}$ values of purified NGF receptor peptides (200,000 and 85,000) were almost identical to those expected from affinity labeling experiments of intact A875 cells, in which two bands of 220,000 and 93,000 were specifically cross-linked to $^{125}$I-NGF. These results suggest that both peptide components of the NGF receptor in A875 cells are purified in this chromatography procedure and remain relatively intact. Affinity labeling of soluble extracts and purified NGF receptor also displayed only two NGF binding peptides, of $M_{r} = 220,000$ and 93,000, again indicating that solubilization and subsequent purification of the NGF receptor results in little alteration of component polypeptides.

When solubilized NGF receptors from membranes of rabbit superior cervical ganglia were characterized by chromatography on Sepharose 6B and sucrose density gradients (Costrini et al., 1979), the receptor, detected by $^{125}$I-NGF binding activity, consisted primarily of a species of $M_{r} = 135,000$. The appearance of a higher molecular weight component was believed by these authors to represent receptor aggregates. When characterized by affinity labeling studies, the NGF receptor of membranes of rabbit superior cervical ganglia possessed two peptides specifically labeled by $^{125}$I-NGF using the cross-linking agent N-hydroxysuccinimidyl-4-azobenzoate (Massague et al., 1981). After subtraction of the molecular weight of NGF, the peptides possessed $M_{r} = 130,000$ and 100,000. The authors suggested that the $M_{r} = 100,000$ species resulted from degradation of the $M_{r} = 130,000$ species. Recently, intact cells and membranes of PC12 cells affinity labeled with $^{125}$I-NGF revealed three specifically labeled peptides, of $M_{r} = 98,000$, 138,000, and 190,000. While the $M_{r} = 98,000$ species appears to be in greater abundance than the higher molecular weight species, the relationship between these two species is at present unclear. The presence of a lower molecular weight species in intact A875 cells, and its membranes, and, in the present study in solubilized receptors, indicates that it may not be a product of artifactual proteolysis occurring during purification and that this lower molecular weight peptide represents a major component of the A875 NGF receptor. The presence of a higher molecular weight component in purified receptor in the absence of cross-linking to $^{125}$I-NGF would suggest that this peptide is also a component of the NGF receptor, and does not result from cross-linking of two $M_{r} = 93,000$ peptides during the photoaffinity labeling procedure.

The availability of microgram quantities of highly purified, functional NGF receptor will provide sufficient amounts of receptor for studies which have thus far been very difficult, if not impossible, with impure soluble extracts. A more detailed knowledge of NGF receptor structure and function will ultimately permit an understanding of the interaction of the receptor with other membrane and intracellular components. Studies are in progress in this laboratory to determine the physical properties of the receptor and study its binding characteristics before and after reconstitution into lipid vesicles. Preliminary evidence indicates that the receptor may contain a kinase activity related to its function. In addition, partially purified receptor is being utilized to produce antireceptor antibodies, which have not been available thus far for this receptor.

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