Nerve Growth Factor Receptor from Rabbit Sympathetic Ganglia Membranes

RELATIONSHIP BETWEEN SUBFORMS*

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Rozenn N. Kouchalakos† and Ralph A. Bradshaw
From the Department of Biological Chemistry, California College of Medicine, University of California, Irvine, California 92717

The receptor for nerve growth factor (NGF) was purified from Triton X-100 extracts of sympathetic ganglia membranes by affinity chromatography on NGF-Sepharose. Elution of purified receptor was accomplished at pH 5 in the presence of 1 M NaCl. Sodium dodecyl sulfate gel electrophoresis of the purified io-
dinated receptor showed three major bands at \( M_r = 126,000, \ M_r = 105,000, \) and \( M_r = 81,000. \) Affinity labeling of the purified receptor using \(^{125}\)I-NGF and the photoreactive agent \( N\)-hydroxysuccinimidyl-p-azido-
dobenzoate resulted in two major cross-linked com-
plexes corresponding to \( M_r = 135,000 \) and \( M_r = 110,000. \) This labeling pattern is similar to that ob-
served with sympathetic ganglia membranes (Ma-
assague, J., Guillette, B. J., Czech, M. P., Morgan, C. J., and Bradshaw, R. A. (1981) J. Biol. Chem. 256, 9419–9424) and indicates that these two forms do not arise from the cross-linking procedure. Reaction of the photoaffinity labeled NGF receptors with increasing amounts of trypsin resulted in a progressive decrease in the high molecular weight complex with a concomi-
tant increase in the low molecular weight form. When the larger complex was isolated by electroelution from a sodium dodecyl sulfate gel and treated with trypsin, a species corresponding to \( M_r = 100,000 \) was gener-
ated. These observations are best explained by a pre-
cursor-product relationship for the two NGF receptor species of sympathetic neurons.

Nerve growth factor (NGF)\(^1\) is a polypeptide hormone required for the growth and differentiation of sympathetic and embryonic sensory neurons (1–9). Although the molecular basis for the action of NGF remains to be elucidated, the first step in the chain of events leading to the characteristic cellular responses is the binding of NGF to a cell surface receptor (2–
4). Specific NGF receptors have been detected on plasma membranes from superior cervical ganglia neurons (5–7), em-
byronic dorsal root ganglia neurons (6, 8, 9), and several established cell lines such as rat pheochromocytoma (PC12) cells (10, 11), human melanoma cells (12), and human neu-
roblastoma cells (13).

Some progress has been made in the molecular characteri-
ation of NGF receptors. Hydrodynamic measurements of the detergent-solubilized NGF receptor of superior cervical ganglia
first revealed an asymmetric membrane protein of approx-
imately 135 kDa (14). Covalent cross-linking of the NGF
receptor to \(^{125}\)I-NGF in the same tissue (15), as well as sensory
neurons (16, 17) and PC12 cells (17), yielded two major complexes corresponding to molecular masses of 100–110 and 143 kDa (neurons) or 158 kDa (PC12 cells). Western blots of mouse melanoma membrane proteins identified two NGF
receptor species of 100 and 130 kDa (18). In contrast, chemical
cross-linking (19, 20), characterization of the purified receptor (21), and immunoprecipitation with monoclonal antibodies (22) revealed a single major NGF receptor species of 75–85
kDa in human melanoma cells (A875). However, A875 mela-
noma cells are not responsive to NGF, in contrast to the other
tissues that have been examined.

Steady-state binding of \(^{125}\)I-NGF to sensory neurons (9),
sympathetic neurons (7), and PC12 cells (11) demonstrated the presence of two classes of NGF receptors differing in their affinity for NGF. In PC12 cells, the two NGF receptor popu-
lations differ in their rates of dissociation of bound NGF, trypsin sensitivity, and solubility in Triton X-100 (23). Hos-
ang and Shooter (17) identified the 158-kDa cross-linked species of PC12 cells as the high affinity, trypsin-resistant form of the NGF receptor (from which bound NGF dissociates slowly) and the 100-kDa species as the low affinity, trypsin-resistant NGF receptor (showing fast dissociation). This iden-
tification is supported by the observation that cells displaying both kinetic classes of NGF receptors show both cross-linked complexes (15, 17), whereas cells displaying only low affinity receptors show little or none of the 143–158-kDa cross-linked complex (17, 19).

The relationship between the two major NGF receptor
species of neurons and PC12 cells is speculative. Massague et
al. (15) suggested a conversion of the 143-kDa labeled receptor
into the 112-kDa species by limited proteolysis, based on
peptide mapping experiments. On the other hand, Hosang and Shooter (17) observed that the relative amount of labeling of the two complexes in PC12 cells did not vary in the presence of protease inhibitors and suggested that the 158-kDa species was not converted to the 100-kDa species, at least by a common protease.

There are several possible relationships between the two
NGF receptor species: 1) the larger species could be the result of cross-linking of the smaller receptor with another protein; 2) the smaller entity could be a proteolytic product of the larger one; or 3) the two species could be genetically unrelated.
Since the identification of the receptor subspecies has been
dependent on covalent cross-linking experiments, it is also possible that the differences are introduced during this modification. The experiments presented in this report were designed to test these possibilities and to ascertain the nature of the receptor responsible for biological activity. We report the purification of NGF receptor from superior cervical ganglia and the biochemical characterization of the purified receptor by direct iodination, photoaffinity labeling, and limited proteolytic digestion. Our results strongly suggest that both receptor forms occur in situ and that they share a precursor-product relationship in keeping with previous observations (15).

MATERIALS AND METHODS

Reagents—NGF was purified from adult male mouse submandibular glands, obtained from Biolog, St. Paul, MN, according to Bocchini and Angeletti (24). 125I-NGF was prepared by a modification (15) of the Bolton-Hunter technique (25). Superior cervical ganglia were obtained freshly frozen from Pel-Freez. Cyanogen bromide-activated Sepharose was purchased from Pharmacia. NGF was coupled to the activated Sepharose according to the manufacturer's instructions. 35S-Bolton-Hunter reagent was obtained from New England Nuclear. Na125I was from ICN. High purity, low peroxide Triton X-100 was from Pierce. Phenylmethanesulfonyl fluoride, pepstatin, leupeptin, chloramine T, 8-octyl glucoside, poly-L-aspartic acid, and diphenylcarbamyl chloride-treated trypsin (Type XI) were from Sigma. EDTA, 0.1 mM pepstatin, and 0.1 mM leupeptin were present throughout the microsome preparation. To solubilize NGF receptors, the microsomes, at a protein concentration of 1 mg/ml in HEPES (10 mM) saline buffer, pH 7.5 (HBS), with protease inhibitors, were incubated with 1% (v/v) Triton X-100 for 1 h at 0°C followed by centrifugation at 100,000 × g for 1 h at 4°C. The supernatant fraction was diluted to adjust the final detergent concentration to 0.25%. NGF receptors were purified from the supernatant fraction by affinity chromatography on NGF-Sepharose. The Triton X-100 extract was incubated in batches with NGF-Sepharose for 12 h at 4°C with gentle stirring on an orbital shaker (for an extract corresponding to 20–200 superior cervical ganglia, 1 g of NGF-Sepharose was used). The NGF-Sepharose beads were poured into a small column and washed successively with 2 column volumes of 0.1% Triton X-100 in HBS with protease inhibitors, 6 column volumes of 1 M NaCl in 0.1% Triton X-100 in HBS, and finally 10 column volumes of 1% β-octyl glucoside in HBS. The purpose of the last wash was to eliminate the Triton X-100 and the protease inhibitors which interfere with the subsequent iodination of the purified receptors. The receptors were desorbed from NGF-Sepharose with a sodium acetate (50 mM) buffer, pH 5, containing 1 M NaCl and 1% β-octyl glucoside. Fractions, 0.5 ml, were collected and immediately neutralized with 0.2 M NaOH. Aliquots (10–100 μl) of the fractions were assayed for 125I-NGF binding activity according to Costolini and Bradshaw (26). The NGF receptor-containing fractions were pooled and kept at −70°C. In order to check the specificity of the affinity matrix for NGF receptor, the affinity chromatography was performed as described above except that an additional wash with buffer containing unlabeled NGF was introduced prior to the desorption step between the second wash (1 M NaCl in buffer, pH 7.5) and the last one (removal of Triton X-100). The buffer used consisted of 3–5 M HBS containing 1 mg/ml NGF (or cytochrome c) and 0.1% Triton X-100.

Iodination of NGF Receptors—Aliquots, 50 μl, of NGF-Sepharose eluate containing 125I-NGF binding activity were incubated with 100 μCi of Na125I and 200 μg of chloramine T for 5 min. The reaction was quenched with 500 μg of potassium meta-bisulfite and 500 μg of sodium iodide. To eliminate excess reagents, the reaction mixture was diluted with 1 ml of HBS containing 1% β-octyl glucoside and 0.2% poly-L-aspartic acid (15,000 average molecular weight) as a carrier, and concentrated to 50 μl with a Centricon-30 microconcentrator (Amicon).

Photoaffinity Labeling Protocol—The purified NGF receptors were labeled with 125I-NGF using the photoactivatable cross-linker HSAB by a modification of the method of Massague et al. (15). Aliquots of the eluate of the NGF-Sepharose column, 10–50 μl, were incubated for 60 min at 23°C with 0.8 nM 125I-NGF in the presence or absence of 0.4 μM unlabeled NGF in a total volume of 200 μl (dilutions up to volume were made with HBS containing 0.1% Triton X-100). At the

![Figure 1. Affinity chromatography of rabbit sympathetic ganglia NGF receptor on an NGF-Sepharose column.](image)

![Figure 2. Sodium dodecyl sulfate polyacrylamide electrophoresis of purified iodinated NGF receptors.](image)
end of the incubation period, HSAB, freshly dissolved in dimethyl sulfoxide, was added at 50 μM final concentration. The reaction mixture was incubated for 4 min on ice in the dark and then transferred to a quartz cuvette and photolyzed for 8 min at 4 °C using a short wave-length lamp (UV mineralight lamp UVS-11). The reaction was stopped by adding 10 volumes of 10 mM Tris, pH 7.0, with 0.1% Triton X-100. Excess reagents were in part eliminated by concentrating the reaction mixture to 50 μl with a Centricon-30, diluting the concentrate to 1 ml with 0.28% SDS and concentrating again. The final concentrate was retained for electrophoresis. When samples of photoaffinity cross-linked receptor were treated with trypsin, the above protocol was followed up to the first concentration. Digestion with a suitable trypsin concentration was performed on aliquots of the first concentrate at 23 °C for 30 min. The reaction was quenched by addition of 1% SDS and 50 mM dithiothreitol followed by boiling for 1 min. Samples treated in this fashion were directly analyzed by SDS electrophoresis.

Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis—Iodinated and photoaffinity labeled NGF receptors were diluted with an equal volume of sample buffer (20 mM Tris, pH 6.8, 2% SDS, 2 mM dithiothreitol, 15% sucrose, 0.01% bromphenol blue) and boiled for 1 min prior to electrophoresis. Gel electrophoresis was performed according to Laemmli (27), using a 3% stacking gel and a 6% separating gel. After electrophoresis, the gels were stained in 0.025% Coomassie Blue, 25% 2-propanol, 10% acetic acid, and destained in 10% acetic acid. Autoradiograms were obtained from the dried gels after exposure to Kodak X-Omat AR film, using a DuPont Lightning Plus enhancing screen. Molecular weight standards were myosin (Mr = 200,000), β-galactosidase (Mr = 116,000), phosphorylase b (Mr = 93,000), bovine serum albumin (Mr = 66,000), and ovalbumin (Mr = 46,000).

Electrophoresis of High Molecular Weight Affinity Labeled NGF Receptor—After electrophoresis of the photoaffinity labeled NGF receptors, the polyacrylamide SDS gel was briefly stained and the area of the gel where the 130-135-kDa NGF receptor species was expected to be found was cut out of the gel and sliced into small pieces. Electrophoretic elution of the photolabeled NGF receptor species was performed using the electrophoretic tank and the elution cell designed by Hunkapiller et al. (28). The gel pieces were transferred to the bottom of an elution cell on top of a membrane with a 3% stacking gel and a 6% separating gel. The mixture was carefully overlaid and the elution cell filled with running buffer (0.2% SDS in 0.01 M sodium bicarbonate). Electrophoresis was performed at 70 V for 24 h, during which the running buffer was recycled. The receptor species was recovered by precipitation with 9 volumes of ice-cold methanol after addition of a few micrograms of bovine serum albumin as carrier protein. The pellet was dissolved in 0.1% Triton X-100 in HBS. Half of the receptor solution was submitted to trypsin digestion and the other half was reserved as a control.

RESULTS

Purification of NGF Receptor from Sympathetic Ganglia—NGF receptors were purified from Triton X-100 extracts of rabbit superior cervical ganglia microsomes. During the purification, the receptor was detected with a soluble 125I-NGF binding assay making use of the differential precipitation of the NGF-receptor complex and unbound NGF with polyethylene glycol (26). Binding of the Triton X-100-solubilized NGF receptors to NGF-Sepharose was performed in batches thus allowing 90% of the binding activity of the Triton X-100 extracts to be adsorbed (data not shown). After adsorption, the beads of NGF-Sepharose were packed in a column and eluted (Fig. 1). No binding activity was released from the column by the initial wash with the high salt, pH 7.5 buffer, indicating that the receptor was tightly bound. This step allowed the removal of most contaminating proteins nonspecifically adsorbed to NGF-Sepharose. The column was then extensively washed with a β-octyl glucoside buffer in order to remove the Triton X-100. This detergent exchange was necessary for the later iodination of the eluted NGF receptors (see below). NGF receptor was desorbed with a sodium acetate buffer, pH 5, containing 1 M NaCl and 1% β-octyl glucoside. Approximately 30% of the binding activity of the Triton X-100 extracts was recovered in the sodium acetate eluate of the NGF-Sepharose. The overall loss of binding activity during the purification procedure is probably due in part to an effectively irreversible association of some of the NGF receptors with the NGF-Sepharose and in part to the progressive loss of binding activity of the receptors observed at 4 °C.

NGF receptor was totally desorbed by a wash containing NGF, as demonstrated by the absence of binding activity in the subsequent elution with the sodium acetate buffer (Fig. 1). On the other hand, cytochrome c was unable to displace the receptor, indicating that adsorption of NGF receptor to the NGF-Sepharose was specific.

![Fig. 4. Effect of digestion with trypsin of photoaffinity labeled NGF receptors.](image-url) Purified NGF receptors, incubated with 0.8 nM 125I-NGF in the presence (lanes B, D, F, and H) or absence (lanes A, C, E, and G) of 0.4 μM NGF, were cross-linked with HSAB. The samples were then treated with the indicated concentrations of trypsin (Panel A, 0–1 μg/ml; Panel B, 0–20 μg/ml) and electrophoresed in a 6% polyacrylamide gel. Autoradiograms of the dried gels are shown.
were incubated with 0.8 nM species was electroeluted as specified under "Material and Methods" receptor species into a 100-kDa species. Purified autoradiogram of the dried gel is shown.

and submitted again to electrophoresis after digestion with 1 pg/pl eluate for a protein concentration measurement. Quantitation of the presence of Triton X-100 in the original extract, and detergents which would have allowed iodination (β-octyl glucoside, CHAPS, Tween 20) were not as efficient as Triton X-100 in solubilizing the NGF receptor from sympathetic ganglia membranes. Analysis of the proteins eluted in the NGF receptor-containing fractions of the NGF-Sepharose column, but its labeling intensity indicated a much lower affinity for 125I-NGF than the other NGF receptor components. The same specific labeling pattern of the major species was also observed with superior cervical ganglia membranes (15), indicating that the NGF receptor species present in the membrane are solubilized by Triton X-100 and co-purify on NGF-Sepharose. The minor 84-kDa cross-linked species was not visible in the labeling pattern from membranes (15), suggesting that it could also be an artifact of the cross-linking procedure in solution. Alternatively, cross-linking to receptors in the membrane may not have been sensitive enough to detect this component.

Conversion between the Two Major Photolabeled NGF Receptor Species—NGF receptors cross-linked to 125I-NGF were submitted to trypsin digestion with increasing amounts of enzyme, followed by SDS gel electrophoresis and autoradiography (Fig. 4A). The amount of the high molecular weight cross-linked NGF receptor species (M, 130,000 in this gel) decreased relative to the smaller major cross-linked species (M, 105,000) when the trypsin concentration was increased from 0 to 1.0 μg/ml.3 At higher concentrations of trypsin, the larger species totally disappeared, whereas the smaller species remained visible, albeit that it decreased as well, after digestion with 20 μg/ml trypsin (Fig. 4B). These results suggest that the 105-kDa cross-linked NGF receptor species is a proteolytic product of the 130-kDa species. In contrast, the minor 83-kDa cross-linked species remained invariant, suggesting that it was not a proteolytic product of the larger species (Fig. 4A), although the small amounts present initially render this judgement tentative in nature.

In order to observe directly the conversion of 130-kDa species to the 105-kDa form, the larger cross-linked NGF

As observed previously (15), the relative amounts of the two species of NGF receptor are variable from preparation to preparation, and the sample utilized in the experiment contained only trace amount of the lower molecular weight form; that may have occurred because it was purified from freshly prepared membranes as opposed to membranes stored at -70 °C.

3 R. N. Kouchalakos, unpublished observations.
receptor species was electroeluted from an SDS gel, submitted to limited trypsin digestion, and electrophoresed again on an SDS gel (Fig. 5). Indeed, a species corresponding to approximately 100 kDa was generated, suggesting that the 100–110-kDa NGF receptor species observed by direct iodination (Fig. 2) and photoaffinity labeling (Fig. 3) was derived from the 130–135-kDa receptor species by proteolysis.

**DISCUSSION**

A simple method has been devised to isolate NGF receptors from superior cervical ganglia membranes in substantially purified form. This is the first report of NGF receptor purification from a natural target, i.e. tissue responsive to NGF in vivo. Triton X-100 was the most efficient of the detergents tested to solubilize the NGF receptor. The purification consisted of a single round of affinity chromatography on NGF-Sepharose, from which NGF receptors were easily desorbed by decreasing the pH of the eluting buffer to 5.0. Elution at pH 5.0 in the presence of a high concentration of salt from an affinity matrix has also been used successfully in the purification of insulin-like growth factor II receptor (29) and the insulin receptor (30).

The photoaffinity labeling pattern of the purified NGF receptors with I\(^{125}\)I-NGF using the reagent HSAB displayed two major cross-linked NGF receptor species corresponding to molecular masses of 130–135 and 100–110 kDa. This pattern was similar to the one obtained with intact membranes of sympathetic ganglia where two major species of 143 and 112 kDa were observed (15). Clearly, the main receptor species of the membranes were solubilized and purified. The small difference in the estimation of the molecular weight of the larger form could reflect some proteolysis during solubilization and purification in spite of the presence of protease inhibitors; however, electrophoresis of the cross-linked purified NGF receptors and receptors cross-linked in the membrane on the same SDS gel showed that the respective NGF receptor species co-migrated. The difference in the estimated molecular weights reported in these studies herein and previously (15) probably reflects minor technical differences in the SDS gels used.

The photoaffinity labeling patterns of receptors from membranes and in a purified form did differ in the minor cross-linked species observed. Sympathetic ganglia membranes displayed a minor 300-kDa band which was absent in the purified receptors, suggesting that this band could have corresponded to the cross-linking of one of the NGF receptor species with another protein which did not co-purify on NGF-Sepharose. Alternatively, it could have been a dimer of the 143-kDa NGF receptor species which dissociated during the Triton X-100 solubilization. On the other hand, the purified cross-linked NGF receptors displayed a minor 84-kDa species which was not detected in the membrane sample. Whether the 84-kDa species represented a genuine, albeit low affinity, NGF receptor component or simply the artificial cross-linking of I\(^{125}\)I-NGF with the bovine serum albumin used as carrier protein remains to be established.

The studies presented here allow a better understanding of the relationship between the two main NGF receptor species observed after cross-linking. Direct iodination of the purified NGF receptors revealed two species of 126 and 105 kDa which correspond, respectively, to the 130–135- and 100–110-kDa photoaffinity labeled forms. The larger NGF receptor species, therefore, is apparently a single-chain component since the direct isolation experiments eliminate the possibility that the 130–135-kDa species results from cross-linking of the 100–110-kDa species with either dimeric (or even larger aggregates) of NGF or another protein. However, they do not rule out the possibility of a second, noncovalently associated protein that does not dissociate in either the ionic or nonionic detergents utilized in these experiments.

Several lines of evidence support the possibility that the 100–110-kDa NGF receptor species is a proteolytic product of the larger molecule: 1) progressive trypsin digestion of the photoaffinity labeled NGF receptors results in a decrease in the amount of the 130–135-kDa species concomitant with an increase in the 100–110-kDa band, as judged by the labeling intensity (Fig. 4A); 2) the amount of the 130–135-kDa species was greater with NGF receptors purified from freshly prepared membranes than membranes stored at ~70 °C (compare Figs. 3 and 4A); and 3) trypsin treatment of the purified 130–135-kDa species electroeluted from SDS gels produced a 100-kDa fragment (Fig. 5). These data together with the previously reported observation of four proteolytic fragments common to the two NGF receptor species (15) strongly suggest a precursor-product relationship for the two NGF receptor species.

Characterizations of NGF receptors from various sources have led to the description of a number of forms summarized schematically in Fig. 6. These forms can be conveniently...
have led to the description of a number of forms summarized schematically in Fig. 6. These forms can be conveniently grouped into four classes according to their general size: class A, 70 to 81 kDa; class B, 87 to 105 kDa; class C, 120 to 145 kDa; and class D, 190 to 300 kDa. Whether all classes reflect genuine receptor species, as opposed to binding proteins, remains to be proven since all have been identified only through their ability specifically to bind labeled NGF. However, it is significant that class C receptors are consistently present in tissues and cells responsive to NGF yet absent from human melanoma cells which are not stimulated by NGF. Another feature unique to the human melanoma cells is the presence of a uniform population of NGF-binding sites exhibiting low affinity toward NGF (20), in contrast with sympathetic neurons, sensory neurons, and PC12 cells, which all exhibit both high and low affinity NGF-binding sites (7, 9, 11). Since convincing evidence has been presented that NGF-mediated neurite outgrowth, as well as other responses, occurs through interaction with the high affinity binding sites only (31-33), it suggests that these correspond to the class C receptors. This coincides with the findings of Hosang and Shooter (17) who found the photoaffinity labeled receptor species of 158 kDa in PC12 cells to be chase-stable at 0 °C and trypsin-resistant, indicating that it is the receptor from which NGF dissociates slowly. In addition, the 158-kDa species is preferentially labeled at low 125I-NGF concentrations (17, 34), suggesting that it is also the high affinity NGF receptor, i.e. the receptor mediating NGF actions. Thus, the correlation of class C receptor species with responsiveness to NGF (Fig. 6), together with the identification in PC12 cells of the class C species as the high affinity NGF receptor (17), suggests that these are the biologically relevant NGF receptors.

According to the studies presented herein, class B species are derived from class C by limited proteolysis in sympathetic neurons. However, it is important to note that this classification scheme does not establish that receptors of similar molecular weight may not be entirely different from one organism (or tissue) to another. In this regard, the low molecular weight (class A and B) receptors of the human melanoma cell line A875 may not represent true receptors at all. Alternatively, they may have arisen from the receptor gene by truncation, either of the gene itself or its mRNA, analogous to the erbB protein and the epidermal growth factor receptor (35), rather than from proteolysis of a larger form. In the case of the erbB protein, however, the epidermal growth factor-binding domain has not been retained and thus it is not strictly analogous to the possible situation in A875 cells. The relationship of these melanoma receptors or any of the other species of NGF receptors reported to be in the class C group will require more definitive structural data.

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