ABSTRACT

Nerve growth factor (NGF), a hormone-like regulator of sympathetic neuron ontogeny and metabolism, affects its target cells initially by associating with specific plasma membrane receptors. We have solubilized the NGF receptor of adult rabbit superior cervical ganglia (SCG) with the nonionic detergent Triton X-100. The high-affinity equilibrium binding constant of the detergent-extracted receptor is 2 × 10⁻⁹ M. Gel chromatography of the receptor or the ³²P-labeled NGF-receptor complex on a column of Sepharose 6B indicated, in both cases, a single component of an apparent hydrodynamic radius of 71 ± 5 Å. In parallel investigations, we have confirmed the similarity between the hydrodynamic size of the NGF receptor of rabbit SCG and that of the insulin receptor of IM-9 lymphocytes evaluated by similar methods.

Nerve growth factor (NGF) is a hormone-like polypeptide that is responsible for the growth, development, and maintenance of the sympathetic nervous system. As with other substances of related function, it appears that the first step is the association of the factor with a specific recognition site (receptor) on the surface of responsive cells (1). The binding characteristics of this receptor from both sympathetic and sensory neurons have been extensively studied (2–4) and Banerjee et al. (5) have shown that the NGF receptor can be extracted from the cell surface by nonionic detergents without loss of its ligand binding activity. In this report we present the binding characteristics and apparent molecular size of the NGF receptor extracted from rabbit superior cervical ganglia (SCG) with the nonionic detergent Triton X-100.

MATERIALS AND METHODS

Preparation of NGF and ³²P-Labeled NGF (³²P-NGF). 2.5S NGF was prepared from mouse submaxillary glands according to the method of Bocchini and Angeletti (6). ³²P-NGF was prepared by a modification (5) of the Bolton and Hunter (7) technique. A mixture of 5 mCi (1 Ci = 3.7 × 10¹⁰ becquerels) of ³²P-labeled Bolton-Hunter reagent (New England Nuclear; specific activity, 1500 Ci/mmol) and 1 nmol of 2.5S NGF in 10–20 µl of ice-cold 0.1 M borate pH 8.5 buffer was allowed to react for 10 min. Then the reaction was stopped with 100 µl of 0.5 M glycine in borate buffer. Iodinated NGF was separated from other components of the reaction mixture by chromatography on Sephadex G-25 (fine; 1 × 16 cm). ³²P-NGF was 95–98% trichloroacetic acid-precipitable and 90–95% immunoprecipitable. Incorporation of labeled reagent varied from 20 to 40%; specific activities varied from 900 to 2000 cpm/fmol (42–93 µCi/µg; up to 0.5 mol of reagent per mol of NGF). Higher specific activities could be obtained by increasing the reaction time; however, this was attended by damage to the NGF as judged by higher nonspecific binding in the radioreceptor assay.

In order to minimize background and nonspecific radioactivity associated with the soluble NGF receptor assay and to use only chromatographically monocomponent-labeled NGF in studies requiring gel filtration of ³²P-NGF, aliquots of labeled peptide were thawed and passed through a column of Bio-Gel P-100 (1 × 20.0 cm) equilibrated with bovine serum albumin at 1 mg/ml/136 mM NaCl/4 mM KCl/0.05 M Tris-HCl, pH 7.5. Radioactivity eluting in a position corresponding to an approximate molecular weight of 27,000 (50–70% of applied radioactivity) was pooled and used in all experiments. Radioactivity eluting in the void volume consisted of ³²P-NGF which was associated with 3-fold higher nonspecific binding in preliminary NGF receptor assays and was therefore discarded. Fresh ³²P-NGF was prepared every 3 weeks.

Preparation of Soluble NGF Receptor. A plasma membrane-enriched microsomal fraction of mature rabbit SCG obtained frozen from Pel-Freez was initially prepared according to previously described methods (4). To prepare microsomes, 200–400 SCG were Polytron-homogenized at 4°C in 0.32 M sucrose/110 mM NaCl/10 mM CaCl₂ and processed by the serial centrifugation protocol shown in Fig. 1. Microsomes (P₃) were solubilized in 1% (vol/vol) Triton X-100 at room temperature for 30 min; the incubation mixture was then centrifuged at 100,000–300,000 X g (similar results) for 1 hr. The supernatant fraction (S₄) was filtered through a 0.45-µm Millipore filter and then diluted or dialyzed to adjust the final detergent concentration to 0.1%. Protein determinations on S₄ were made according to the Markwell et al. (8) modification of the Lowry method (9).

Assay for NGF Receptor in Triton-Extracted SCG Microsomes. The presence of NGF receptor binding activity in S₄ was determined according to a modification of the method of Banerjee et al. (5). Aliquots of S₄ containing 5–30 µg of protein were incubated with ³²P-NGF for 60 min at 22°C in Heps/Hanks’ medium with 0.1% Triton X-100 and 5 µg of bovine serum albumin per ml in a total reaction volume of 200 µl. The ³²P-NGF–receptor complex was precipitated by addition of 0.5 ml of 0.15% bovine gamma globulin and 21% (wt/vol) polyethylene glycol 6000 in ice-cold 0.05 M Tris-HCl buffer at pH 7.5. The samples were mixed, placed on ice for 15 min, and centrifuged at 3000 × g for 20 min. The supernatant fractions were decanted and the pellets were washed with 2 ml of 8% polyethylene glycol. The radioactivity in the final pellets was determined in a Beckman 300 gamma counter (counting efficiency, 75%). Background binding (5–8% of total counts in the reaction mixture) was determined by carrying out parallel incubations in the absence of extracted protein; nonspecific binding (40–50% of counts in final pellet) was determined by carrying out incubations in the presence of a 1000-fold excess of NGF.

Abbreviations: NGF, nerve growth factor; SCG, superior cervical ganglia.

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Rabbit SCG

1. Homogenize
2. Centrifuge, 1000 X g, 10 min

S1

3. Repeat steps 1 & 2

P1

4. 7700 X g, 10 min

S2

5. 100,000 X g, 60 min

Pl

Pl'

6. 1% Triton X-100, 21°C, 30 min
7. Dilute 1:4, 0°C
8. Centrifuge 1-3 hr, 100,000 X g

S1

Pl

P2

Pl

Triton extracted NGF receptor preparation

9. Filter

FIG. 1. Preparation of plasma membrane-enriched neuronal subfraction and Triton X-100 solubilization of NGF receptor from rabbit SCG.

of unlabeled 2.5S NGF. Nonspecific binding to the receptor-containing extract constituted 20% of the total 125I-NGF binding to the extract.

Chromatography of the NGF Receptor and Determination of the Effective Hydrodynamic Radius (Stokes Radius). The apparent Stokes radius of the NGF receptor in Triton or of the 125I-NGF-receptor complex was determined on a column of Sepharose 6B (0.9 X 58 cm) equilibrated with Hanks' balanced salt solution containing 0.05% Triton X-100, 1 mg of bovine serum albumin per ml, and 20 mM Hepes at pH 7.5. Void and inclusion volumes were determined with blue dextran and Na125I. In later experiments, β-galactosidase was included as an intrinsic marker. Samples for chromatography were prepared by incubating 500 µg of S2 protein with 0.1 nM 125I-NGF in a volume of 0.5 ml for 1 hr at room temperature in the absence or presence of an excess of unlabeled NGF. To determine the Stokes radius of the uncomplexed NGF receptor, 600-1600 µg of S2 protein was chromatographed under identical conditions. Specific 125I-NGF binding activity in the elution fractions was determined by the soluble receptor assay. Control studies included chromatography and assay for NGF receptor activity in 1-mg samples of bovine serum albumin and of a preparation of Triton-extracted human erythrocyte membranes. The Stokes radii of the NGF receptor and ligand-receptor complex were determined graphically according to the method of Laurent and Killander (10). The partition coefficients (Kαv) of a group of standard proteins (see legend, Fig. 4) of known Stokes radius were determined by chromatography under conditions identical to those used for receptor chromatography. These standard proteins were obtained from the literature (11) and were linearly related to (−log Kαv)1/2.

To compare the Stokes radius of the NGF receptor with that of the insulin receptor, IM-9 lymphocytes (12) were kindly provided by James R. Gavin III (Metabolism Division, Washington University School of Medicine). Detergent extracts of these cells were prepared and the Stokes radius of the extracted insulin receptor was determined in a manner similar to that used to determine the size of the 125I-NGF-receptor complex. 125I-Labeled insulin (specific activity, 300 µCi/µg) was prepared according to a modified lactoperoxidase technique (13) using Enzymobeads (Bio-Rad).

Commercially obtained reagents, peptides, and chemicals were as follows: reagent grade Tris, Hepes, beef heart cytochrome c (90-100% pure), bovine serum albumin (RIA grade), polyethylene glycol 6000, and bovine gamma globulin (59%, Cohn fraction II), Sigma; except for β-galactosidase (E. coli, grade IV, Sigma), chromatographic standards (α-dolase, castase, ovalbumin, bovine serum albumin) were obtained from Boehringer Mannheim; porcine insulin, Eli Lilly; Triton X-100, Rohm and Haas; human growth hormone was kindly provided by James R. Gavin III.

RESULTS

Preparation of Triton-Extracted NGF Receptor. Rabbit SCG were selected as a source of NGF receptors after evaluation of several potential sources including embryonic chicken sensory ganglia, mature rat sympathetic ganglia, and porcine adrenal medulla. Although each source contained NGF receptors with affinity binding constants in the 10−10 M range, only mature rabbit SCG tissue could be readily obtained in relatively large amounts and contained NGF receptors in high concentrations. Although the plasma membrane-enriched neuronal subfraction (P3, Fig. 1) used in preparing the soluble receptor preparation contained only 1-2% of the protein in the crude ganglionic homogenate, it had greater than 80% of the receptor binding activity. Triton X-100 at a concentration of 1% (vol/vol) solubilized 50-60% of P3 protein in 30-45 min when the protein concentration was <4 mg/ml; at concentrations of microsomal protein above this, higher detergent concentrations were required for the detergent solubilization.

Binding Characteristics of the Detergent-Extracted Receptor. A typical competitive binding experiment of the Triton-extracted NGF receptor, in which aliquots of Triton extract were incubated with 0.1 nM 125I-NGF alone and in the presence of increasing concentrations of unlabeled NGF, is depicted in Fig. 2 left. Approximately 80% of the labeled NGF that was bound to the detergent extract in the absence of unlabeled NGF could be prevented from binding by the presence of 1 µM NGF; this percentage constitutes the specific binding to the receptor. In contrast to the effects of 1 µM NGF, similar concentrations of porcine insulin, cytochrome c, and human growth hormone were without effect on the binding. The corresponding Scatchard analysis of the competitive binding data is shown in Fig. 2 right. The inset shows the high-affinity region of the curve on a 10-fold expanded abscissa; the equilibrium binding constant, calculated from the slope, is 6.1 X 10−10 M. In numerous experiments, under various conditions, the equilibrium binding constant varied from 2 to 8 X 10−10 M. This range of affinity is similar to that reported for the plasma membrane-associated receptor in sensory (2, 3) and sympathetic ganglia (4).

Chromatography of the Detergent-Solubilized NGF Receptor. The chromatographic behavior of the NGF receptor is depicted in Fig. 3. When the receptor-containing Triton extract was incubated with 0.1 nM 125I-NGF, a small peak of radioactivity eluted at fraction 48, corresponding to a partition coefficient (Kαv) of 0.36, and was clearly distinguishable from the much larger peak of free 125I-NGF. The smaller peak of radioactivity was greatly decreased when the chromatographed incubation mixture contained 1 µM unlabeled NGF or when 125I-NGF was chromatographed alone. These findings suggest that the smaller peak of radioactivity represents the elution position of the 125I-NGF-receptor complex. In order to confirm that this peak corresponds to the receptor, the Triton-extracted protein of SCG containing the NGF receptor was chromat-
FIG. 2. Binding characteristics of NGF receptor extracted from SCG by Triton X-100. (Left) Competitive binding experiment. Detergent-extracted protein (50 µg) was incubated with 0.1 nM [125I]-NGF in 200 µl of incubation medium for 1 hr in absence or presence of increasing concentrations (50 pM to 1 µM) of unlabeled NGF or 1 µM human growth hormone (O), porcine insulin (X), or cytochrome c (Q). The [125I]-NGF–receptor complex was precipitated with polyethylene glycol and gamma globulin. Total [125I]-NGF in the pellet minus background radioactivity is plotted on ordinate and corresponding peptide concentration on the abscissa. (Right) Corresponding Scatchard analysis of the competition curve. Each point is the mean of quadruplicate determinations. (Inset) the abscissa has been expanded 12-fold. $K_v$ is the calculated equilibrium binding constant for the NGF receptor.

Graphed and the elution position of the NGF receptor was determined by application of the soluble receptor assay. As shown in Fig. 3B, a large peak of [125I]-NGF specific binding occurred at fraction 46 ($K_v = 0.34$). A smaller peak of specific activity was occasionally seen just after the void volume and presumably represents aggregated receptor-containing material. In contrast, a comparable peak near or at the void volume was rarely seen in the experiments of the type described in Fig. 3A. The small amount and variable presence of receptor activity at or near the void volume prevented more thorough examination. When samples of bovine serum albumin or Triton-extracted human erythrocyte membrane proteins were similarly chromatographed and evaluated by the soluble receptor assay, no binding activity above background was seen. Taken together, the results of Fig. 3 are complementary and indicate a single elution position for the [125I]-NGF–receptor complex or for the receptor alone.

The relationship between the partition coefficients ($K_v$) and corresponding Stokes radii for the standard proteins used to estimate the size the NGF receptor is shown in Fig. 4. The replicate standard elution positions were essentially identical. When the $K_v$ values of the [125I]-NGF–receptor complex and the receptor alone were applied to this relationship, their apparent Stokes radii were nearly identical; mean (±SD) for five determinations was 71 ± 5 Å. This value was confirmed by the simultaneous chromatography of the receptor and β-galactosidase; specific binding activity eluted just ahead of β-galactosidase which has a Stokes radius of 69 Å (11). The elution behavior of NGF receptor binding activity was unaffected by addition of dithiothreitol (1 mM) to the elution buffer, by decreasing the salt concentration, or by removing the bovine serum albumin from the elution buffer. The recoveries of chromatographed radioactivity and receptor binding activity were approximately 75% and at least 60%, respectively.

The NGF receptor Stokes radius of 71 Å is the same as that reported for the insulin receptor when similarly analyzed (16, 17). Using insulin receptors from IM-9 lymphocytes, we confirmed this value (Fig. 4).

FIG. 3. Receptor and [125I]-NGF–receptor complex elution profiles during gel chromatography on (0.9 × 60 cm) column of Sepharose 6B. (A) Triton-extracted SCG microsomal protein (500 µg) containing the NGF receptor was incubated with 0.1 nM [125I]-NGF in 500 µl of incubation medium without excess unlabeled NGF (A) or with 1 µM unlabeled NGF (O). D, [125I]-NGF alone. After incubation, the entire reaction mixture was chromatographed and the radioactivity in eluted 0.5-ml fractions was determined. $V_o$ and $V_t$ indicate void and total volumes; $K_v$ indicates partition coefficient. (B) Triton-extracted protein (1 mg) was chromatographed as in A without preincubation with [125I]-NGF. Fractions were collected and the NGF receptor in each fraction was measured by the soluble receptor assay.

**DISCUSSION**

These studies provide estimates of the overall size of the intact NGF receptor as it may exist in the plasma membrane of responsive neurons. The most important conclusion that can be drawn from these data is that the NGF receptor is an intrinsic membrane protein with an apparent Stokes radius of 71 ± 5 Å. That the NGF receptor is embedded in the membrane bilayer is indicated by the requirement for detergent to solubilize the protein. In our studies we have noted that, in the absence of detergent, the extracted receptor protein aggregates and precipitates, a property typical of an intrinsic membrane protein. The extent of detergent binding generally parallels the hydrophobic nature of the protein. The requirement of the receptor protein to be associated with detergent in order to remain in solution reflects the probability that the receptor protein is to some extent associated with the lipid bilayer of the neuronal plasma membrane. Because the NGF receptor behaves as an intrinsic membrane protein and binds detergent, it must be concluded that the apparent Stokes radius of the NGF receptor is actually the effective hydrodynamic radius of the receptor plus an uncertain amount of bound detergent. This is true for the Stokes radii values that have been reported for the intrinsic membrane proteins serving as recognition sites (receptors) for acetylcholine (18), prolactin and growth hormone (M. J. Waters and H. J. Friesen, personal communication), insulin (16), and...
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FIG. 4. Relationship between partition coefficient (Kwp) and Stokes radius for the NGF and insulin receptors. Standard proteins were chromatographed on a column of Sepharose G6 under conditions identical to those used in Fig. 3. Standard proteins, their Stokes radii, and the assay methods used were: ovalbumin, 27.3 Å, A at 280 nm; bovine serum albumin (BSA), 55 Å, A at 280 nm and 125I-labeled bovine serum albumin (125I-BSA) radioactivity (13) (identical results); aldolase, 46.0 Å, enzymatic activity [Sigma Technical Bulletin 750 (1976)]; catalase, 52 Å, A at 580 nm; β-galactosidase, 69 Å, enzymatic activity (14). Each point is the mean of two or three determinations. The regression line was calculated by standard statistical methods (15). The $K_w$ of the NGF receptor (Δ) and receptor-ligand complex (O) were applied to the graph. X ± SD = 71 ± 5 Å; n = 5. The size of the 125I-insulin-receptor complex (125I-ins-R) was determined by incubating the detergent extract of IM-9 lymphocytes with labeled insulin and chromatographing the incubation mixture.

The gonadotropins (19). This fact must be considered when attempts are made to determine the sedimentation coefficient, molecular weight, and other physical properties of the receptor protein. As a result of the usually small but definite detergent contribution to overall size and the nonlinear relationship between molecular weight and protein elution position, it is inappropriate to estimate the molecular weight of an intrinsic membrane protein directly by chromatographic means. The amount of detergent bound to the receptor can be calculated by determining the partial specific volume of the receptor.

That the hydrodynamic radii of the insulin and NGF receptors are identical is of interest because of the known similarities between NGF and insulin (20). Although the peptides are structurally homologous, their receptor recognition sites are mutually specific. Our results (Fig. 2) indicate that the detergent-extracted NGF receptor does not recognize insulin, and the work of others indicates the specificity of the insulin receptor for its ligand. Because there are numerous factors that may affect the chromatographic behavior of an intrinsic membrane protein (i.e., asymmetry, detergent binding, and carbohydrate content), this similarity between the insulin and NGF receptors must be interpreted cautiously. The crude detergent-extracted insulin receptor has been reported to have a Stokes radius of approximately 72 Å (16). More recently it has been reported that its apparent size can be decreased to 40 Å by exposing the receptor to insulin (17) or by removing a glycoprotein component (21). Although the observations reported herein have not revealed a subunit structure for the NGF receptor, it is premature to assume that it is composed of only a single polypeptide chain. However, the data presented in Fig. 3, suggest that except for a small amount of apparently aggregated material, there is only a single species of NGF receptor. Identification of only one receptor moiety, at least in terms of size, is consistent with previous reports supporting the view that the nonlinearity seen in Scatchard analyses of binding data is due to negatively cooperative interactions within a single class of NGF receptor molecules (1, 2).

Review of the current literature indicates that many nonionic detergent-extracted hormone receptors have similar apparent Stokes radii, in the range 62–72 Å (16–19). Because the hydrodynamic behavior of membrane proteins is not determined exclusively by the solubilizing detergent (22) and because the apparent size of the receptors does not change significantly when deoxycholate is the solubilizing agent (18), the similarity in Stokes radii is unlikely to be an artifact induced by the Triton X-100. Rather, it may reflect the fact that these hormone and growth factor receptors serve common functions—namely, the transfer of information from the extracellular space to the cell interior. On the other hand, because hydrodynamic behavior is dependent upon many factors, the similarities between the receptors may be purely coincidental and of little significance. Therefore, it follows that this similarity does not necessarily presage any structural homology. As further information about the NGF receptor and other peptide hormone receptors becomes available, the similarities and differences between individual hormone and growth factor receptors should become clearer. This information may allow the receptors to be grouped into classes that reflect their biochemical structures and possibly their mechanisms of action.

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