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BINDING CONSTANTS OF ISOLATED NGF-RECEPTORS FROM DIFFERENT SPECIES

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SUMMARY. It is known that NGF-responsive cells bind NGF at cell surface receptors in a specific and saturable fashion and there are two separate kinds of receptor-ligand binding interactions as judged by Rosenthal analyses. Following isolation of nerve growth factor receptors from embryonic chicken sensory ganglia, rat pheochromocytoma cells and human neuroblastoma cells, equilibrium binding studies were carried out and two different equilibrium binding constants similar to that described for whole cells were determined. This evidence is consistent with the hypothesis that there are two different receptors for NGF which have been conserved.

The interactions of biologically active proteins with appropriate target cell receptors and the subsequent physiological responses are unique and complex events. The binding of NGF to neuronal cells is not well understood. In responsive target cells, NGF is capable of inducing biochemical and morphological changes, the most dramatic of which are increased neuronal survival and outgrowth of functional neurites (1-3). NGF binding to receptors has been demonstrated for both sensory and sympathetic ganglia in vitro (4-10) as well as for several transformed cell lines (11-15). The receptor on rabbit sympathetic ganglia has been found to be a protein of 130,000 daltons (16). Similar receptors have been demonstrated for two other cell types, a rat pheochromocytoma clone that responds to NGF and a human melanoma line that does not (17,18).

Abbreviations: DRG, dorsal root ganglia; GPP, glycoprotein pool; NGF, nerve growth factor; NGFR, nerve growth factor receptors; NP40, noniodet-P40; PEG, polyethylene glycol; PBS, phosphate buffered saline.

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When NGFR binding equilibrium studies are performed using sensory or sympathetic neurons, two distinctive dissociation constants can be determined (4-6). The existence of two dissociation constants has been interpreted in two ways: 1) there are two distinct receptors with different NGF binding properties; or 2) the observed binding heterogeneity is due to changes in receptor characteristics induced by the binding of NGF to an increasing proportion of sites in an otherwise homogeneous receptor population. The former interpretation agrees with the findings of Ishii and Sonnenfeld who have shown that in NGF responsive cell lines low affinity receptors are not necessary for neutire outgrowth (11). Thus, it would be important to determine if the two different binding constants are consistently present in responsive cell lines from different species and whether or not the Kd's are similar. In this paper, the equilibrium binding characteristincs of isolated receptors from a variety of cell types from different species are determined. The assay is performed on detergent solubilized cell lysates in order to remove variables, such as receptor internalization and lateral membrane movement, from the ligand-receptor interactions. The studies took advantage of the fact that NGFR are glycoproteins that bind to lentil-lectin, thus, allowing a many-fold purification of receptors from a relatively crude cell lysate as an initial step. The binding equilibrium data obtained suggests that there are two dissociation constants among NGF receptors present in the NGF-responsive cells tested and that the Kd constants obtained are similar for the different cell types.

MATERIALS AND METHODS

Cells. Sensory ganglia were removed from 8-9-day-old embryonic chickens and were dissociated into a single cell suspension as described elsewhere (5). The human neuroblastoma cell line LAN-1 and the rat pheochromocytoma cell line PC12 were maintained in logrithmic phase of growth in plastic tissue culture flasks, appropriate media (LAN-1 RPMI 1640 supplemented with 20% fetal calf serum; PC12 DMEM supplemented with 10% fetal calf serum and 5% horse serum).

Solubilization and Isolation by Lectin Chromatography. Cells to be studied were lysed in 0.5% NP40 in PBS and incubated on ice for 1 hour. The lysate was then centrifuged 15 minutes at 13,000 x g to remove nuclei. The

supernatant was immediately applied to a lentil-lectin Sepharose column. The column was then extensively washed with 0.25% NP40-PBS and glycoproteins specific for lentil-lectin were eluted with 0.5M alpha-methy mannoside - 0.25% NP40-PBS. This GPP was then concentrated by vacuum dialysis.

Receptor Binding Assay. NGF was iodinated via lactoperoxidase as described elsewhere (4). Specific activity was 2000-4000 cpm/fmole with 90-100% of the counts trichloroacetic acid precipitable. Cells were washed 3 times in PBS and resuspended at 5 x 10^7/ml in the same buffer. Five millicuries of Na ^{125}-I (Amersham, Arlington Heights, IL), 50 µg of lactoperoxidase (Sigma Chemical Co., St. Louis, MO) and 10 µl of 0.03% H_2O_2 were added at 0 and 15 min to this suspension. The reaction was stopped by addition of sodium azide. Soluble receptor binding assays (20,21) were carried out on aliquots before and after chromatography on a lentil-lectin bound Sepharose column. Samples were incubated for 90 minutes at room temperature with 3.0 x 10^{-12} to 3.8 x 10^{-9} M ^{125}-I-beta-NGF in PBS containing 0.5% NP40 and 2 mg/ml cytochrome c. Nonspecific binding was determined with the addition of 10^{-6} M unlabeled beta-NGF. At the end of the incubation time, samples were cooled at 4 degrees and then 0.5 ml of cold 21% PEG in PBS containing 1.5 mg/ml gamma globulin (rabbit or bovine) was added. The samples were incubated at 4 degrees for 15 min and centrifuged at 1900 x g for 20 min. The supernatant was removed, the pellet washed with 8% PEG in PBS and rapidly centrifuged. The supernatant was removed and the pellet assayed for radioactivity. Typical nonspecific binding was from 50-60%. Non-specific binding was determined at each concentration of ^{125}-I-beta-NGF used and subtracted from the total binding to obtain specific binding. Equilibrium dissociation constants (Kd) were determined using a Rosenthal plot (27).

RESULTS. DRG were removed from 8-9-day-old chicken embryos, dissociated and the receptor isolated as described in Materials and Methods. When NGF binding activity was monitored for the lectil-lectin Sepharose column non-adherent and adherent fractions, approximately 95% of the recovered binding activity was in the adherent GPP (Table 1). This is striking because following iodination of dissociated embryonic chicken sensory

TABLE I
RECOVERY OF NGF-RECEPTOR (NGFR) BINDING ACTIVITY
FROM THE LENTIL-LECTIN SEPHAROSE COLUMN

	Percent 125-I-Labelled Protein Bound to Column	Percent Total NGFR Binding Activity Recovered	Percent NGFR Binding Activity in GPP
Sensory Neurons (chicken)	3.5	79 ± 8	93 ± 4
Pheochromocytoma PC12 (rat)	4.2	56	83 ± 3
Neuroblastoma LAN-1 (human)	3.5	60 ± 3	89 ± 7

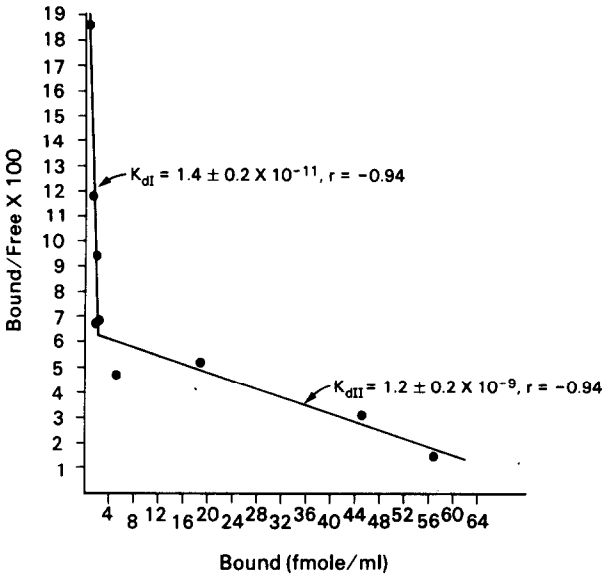

FIGURE 1 Soluble receptor binding assays for NGFR from DRG were carried out as described in Materials and Methods. After data was corrected for nonspecific binding, it was analyzed using a Rosenthal (Scatchard) plot.

ganglia cell membrane surface proteins with ^{125}-Iodine (22-24), only 3-4% of the iodinated proteins adhere to the lentil-lectin columns. Thus, there is a minimum 25-fold enrichment of receptors by lectin binding chromatography (Table 1).

Lectil-lectin adherent GPP were obtained and binding assays carried out to determine the equilibrium dissociation constants (Kd) for NGFR (Figure 1). The binding is consistent with the presence of a high affinity receptor with a Kd of 1.4 x 10^{-11} M and a low affinity receptor with a Kd of 1.2 x 10^{-9} M[1]. Thus, there are two receptors with different equilibrium dissociation constants present in the eluted GPP of solubilized sensory ganglia.

Due to the low amounts of receptor material present, there are few comparisons of the properties of NGFR from different cell types or species. We felt that the lectin enrichment step would provide a unique opportunity for making such comparisons. Firstly, it would give some information

1. The Kd's obtained after correcting for the presence of more than one receptor (H. A. Feldman (1972) Anal. Biochem. 48, 317-338) are not significantly different than those obtained from the Rosenthal plot and are the same as found on whole cells.

regarding the carbohydrate moiety associated with NGFR; and secondly, if the NGFR from all cells bound to lectins, it would yield an enriched pool of NGFR for further purification.

The soluble receptor assay was used to determine the dissociation constants of NGFR in the NGF responsive cell lines PC12, a rat pheochromocytoma, and the LAN-1, a human neuroblastoma. Again, NGFR from these cells were enriched by using lectin chromotography (Table I) and when binding assays were performed on the GPP from PC12 and LAN-1 cells, similar binding profiles as well as Kd's were obtained (Table II). Since 100% of the binding activity is not recovered in the GPP, it is not possible to get an accurate determination of the number of receptors per cell. However, the ratio of high affinity to total binding can be estimated. The ratio of type I to total binding is the same (approximately 4%) for all cell types and is similar to that found in DRG cells (5,6).

DISCUSSION

These studies demonstrate that both high and low affinity NGFR are glycoproteins capable of binding to lentil-lectin and that the determination of two different Kd's is a consistent finding for NGFR on cells from different species, only one of which, the chicken embryonic

TABLE II
EQUILIBRIUM DISSOCIATION CONSTANTS FOR NGF-RECEPTOR COMPLEXES FROM DIFFERENT CELL TYPES

Cell Line	Equilibrium Dissociation Constants[1] TYPE I	TYPE II
Sensory Neurons (chicken)	1.2 ± 0.3 x 10-11M*	1.1 ± 0.2 x 10-9M#
Pheochromocytoma PC12 (rat)	3.8 ± 0.8 x 10-11M*	5.0 ± 2.6 x 10-9M#
Neuroblastoma LAN-1 (human)	5.7 ± 1.7 x 10-11M*	5.5 ± 1.5 x 10-9M#

[1]Numbers are the average ± S.E.M. of 2-5 trials and each experiment was performed twice. (*) and (#) indicate these slopes from the Rosenthal analyses that are not significantly different for different cell types ($p > 0.05$).

tissue, requires NGF for survival. Thus, any hypothetical explanation of the role of the NGFR with regards to its biological action should address this issue. It is likely that the use of a solubilized receptor assay on partially purified GPP removes receptor clustering as a major explanation for the two observed Kd's. The simplest explanation is that there are two structurally distinct NGFR. Indeed, preliminary data on NGFR isolated from rat pheochromocytoma (PC12) and chicken sensory ganglia would suggest that there are at least three different molecular weight species of NGFR[2]. We cannot at this time determine what roles both receptors play in NGF action. However, it is interesting that both cells previously exposed to NGF, chicken sensory ganglia, and cells naive to NGF and that do not require NGF to survive, PC12 and IAN-1, display similar NGFR characteristics.

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