Modification of Nerve Growth Factor Receptor Properties by Wheat Germ Agglutinin*

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PC12 is a nerve growth factor (NGF) responsive cell line which exhibits two classes of NGF receptors distinguishable by different kinetic rate constants, sensitivity to trypsin and resistance to Triton detergent solubilization. Whereas incubation of PC12 cells with wheat germ agglutinin (WGA) prior to addition of 125I-NGF inhibits binding of NGF to both classes of receptors, treatment with WGA subsequent to incubation with NGF does not inhibit NGF binding but causes the class of NGF receptors which exhibit rapid or "Fast" dissociation kinetics prior to lectin treatment to be converted to the form which exhibits "Slow" dissociation kinetics. This WGA-mediated receptor conversion is lectin specific, blocked by N-acetyl-D-glucosamine, occurs at similar rates at 4 and 37 °C, and is not impaired by a metabolic poison. NGF receptors converted by WGA, like pre-existing Slow receptors, are resistant to trypsinization and remain associated to Triton X-100 extracted "cytoskeletons." Very similar results were obtained for NGF receptors on a human melanoma cell line A875. These results suggest that Fast and Slow receptors are two interconvertible forms of a single protein, rather than distinct proteins. The significance of the generality of these properties for NGF receptors from diverse species and cell types is discussed.

Nerve growth factor is a polypeptide which is involved in regulation of growth and differentiation of sensory and sympathetic neurons (1, 2) and probably several other tissues of neural crest origin (3, 4). Our understanding of the mechanism of NGF action has been facilitated by the utilization of the rat pheochromocytoma cell line PC12 isolated and described by Greene and Tischler (5). The response of this clonal line rat pheochromocytoma cell line PC12 isolated and described occurs at similar rates at 4 and 37 °C, and is not resistant to inactivation by low concentrations of tryptsin. Furthermore, NGF bound to Slow receptors is associated with Triton X-100-insoluble cytoskeletal structures whereas NGF bound to Fast receptors (and presumably the receptors themselves) are solubilized by Triton detergent. While Landreth and Shooter (13) proposed that NGF induced heterogeneity in an initially homogeneous receptor population by causing the conversion of a fraction of receptors from Fast to Slow behavior, Schechter and Bothwell (14) found that treatment of cells with trypsin prior to exposure to NGF preferentially eliminated the Fast class of receptor activity, and, therefore, concluded that receptor heterogeneity is not induced by NGF binding but exists prior to exposure to NGF.

The initial event of NGF's mechanism of action is the binding of NGF to specific plasma membrane receptors, a common feature of all polypeptide hormones. Binding studies utilizing biologically active 125I-NGF have detected receptor heterogeneity on sensory and sympathetic ganglion cells as well as on PC12 cells (9-14). Chick embryo sensory and sympathetic neurons express two classes of receptors with similar rate constants of NGF association but differing equilibrium binding constants and rate constants for NGF dissociation (10). PC12 cells also express two classes of receptors which, however, exhibit similar equilibrium binding constants but differ in kinetic rate constants for NGF association and dissociation (13, 14). The two receptor classes are most easily differentiated by their dissimilar rates of release of bound 125I-NGF following addition of a large excess of unlabeled NGF. Schechter and Bothwell (14) introduced the terminology of Fast receptors, denoting the majority of receptors with rapid rates of NGF association and dissociation, and Slow receptors, denoting the 20% of receptors which exhibit relatively slower rates. The two receptor classes are also differentiated by the fact that Slow receptors are more resistant to inactivation by a single protein, rather than distinct proteins. The significance of the generality of these properties for NGF receptors from diverse species and cell types is discussed.

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The abbreviations used are: NGF, nerve growth factor; WGA, wheat germ agglutinin; Hepes, N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid; PBS, phosphate-buffered saline; PBS-CMF, Dulbecco's phosphate-buffered saline with Ca++ and Mg++ omitted.
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100-insoluble cytoskeleton. These data show that NGF receptor heterogeneity on PC12 cells can be eliminated in a reversible fashion by the binding of a particular lectin via specific carbohydrate recognition. In addition, we demonstrate that NGF receptors on human melanoma A875 cells exhibit receptor heterogeneity which is similarly influenced by wheat germ agglutinin and show that the lectin-induced receptor interconversion occurs in membrane preparations from A875 cells but not from PC12 cells.

EXPERIMENTAL PROCEDURES

Materials

Trypsin, soybean trypsin inhibitor, lectins, monosaccharides, bovine serum albumin, lactoperoxidase, phenylmethylsulfonyl fluoride, tosylamide-2-phenylethyl chloromethyl ketone, Repes, Triton X-100, colchicine and cytochalasin B were purchased from Sigma. Na<sup>252</sup>I was obtained from Amersham, RPMI 1640 medium from Gibco, horse serum and newborn calf serum from North American Biological Associates or Flow Laboratories, Inc. NGF was prepared as described previously (15, 16) and was radiodinated by lactoperoxidase according to Sutter et al. (10), and was used at a specific activity of 75-100 cpm/pg (2.0-2.5 × 10<sup>8</sup> cpm/mol of 26,000-dalton NGF dimer), unless otherwise specified.

Methods

Cell Culture—Rat PC12 cells, isolated and described by Greene and Tischler (5), were cultured in RPMI 1640 medium containing 5% newborn calf serum and 10% horse serum on polystyrene dishes (Falcon) at 37 °C in a humidified atmosphere of 5% CO<sub>2</sub> and 95% air. Cells were subcultured every 3-4 days. Human melanoma A875 cells were obtained from the laboratory of George Todoro (7) and were cultured like PC12 cells except that the supplement consisted of 10% fetal calf serum.

Plasma Membrane Preparation—Cells were washed three times with Dulbecco's phosphate-buffered saline with Ca<sup>2+</sup> and Mg<sup>2+</sup> omitted, resuspended in PBS/CMF containing 2 mM phenylmethylsulfonyl fluoride and 0.2 mM tosylamide-2-phenylethyl chloromethyl ketone at 10<sup>6</sup> cells/ml, and lysed by rapid passage through a 26-gauge needle. The extent of cell lysis was monitored by microscopic examination. One-half volume of 60% sucrose in PBS/CMF was added, and the lysate was then centrifuged at 500 × g for 5 min to remove whole cells and nuclei. The clarified lysate was layered on a discontinuous gradient of 35, 45, and 50% sucrose, centrifuged at 23,000 × g for 30 min at 4 °C, and the band at the 30-35% interface was harvested. This microsome fraction was pelleted at 23,000 × g for 2 h and resuspended in 20 mM Hepes, pH 7.4, containing 10 mM MgCl<sub>2</sub> and 2 mM phenylmethylsulfonyl fluoride. Protein concentration was adjusted to 1 mg/ml.

NGF-binding Assay—Cells were harvested by gentle trituration, washed twice with Dulbecco's PBS and resuspended in PBS containing 0.1% serum albumin, and 0.2% glucose at a concentration of 1 × 10<sup>6</sup> cells/ml. Binding of <sup>125</sup>I-NGF was measured essentially according to Schachter and Bothwell (14). Briefly, cells were incubated with <sup>125</sup>I-NGF for the appropriate duration, and 100-μl aliquots were layered onto 200 μl of PBS containing 0.1% serum albumin and 0.1% sucrose in 400-μl microfuge tubes. Cell-bound <sup>125</sup>I-NGF was separated from free by centrifugation in a Beckman microfuge for 1 min. Microfuge tubes were frozen in dry ice-ethanol, and the tips containing all pellets were cut off and placed in 2 ml of 40% sucrose in 400-μl microfuge tubes. Cell-bound <sup>125</sup>I-NGF was consistently less than 5% of total binding and is defined as the amount of <sup>125</sup>I-NGF which associates with cells in the presence of 10 μg/ml (50-fold excess) of unlabeled NGF. All binding measurements were done in triplicate and the data is presented as the mean with standard deviations.

NGF receptors which exhibit slow dissociation kinetics were quantitated by associating <sup>125</sup>I-NGF (20 ng/ml) with cells, adding 500-fold excess unlabeled NGF (19 μg/ml), and incubating for 30 min at 0 °C, pelleting and then counting the remaining <sup>125</sup>I-NGF associated with cells as described above. This procedure dissociates all labeled NGF from receptors exhibiting fast kinetics, whereas labeled NGF bound to Slow receptors remains cell associated. Binding studies done using plasma membrane preparations were performed similarly except that free and membrane-bound <sup>125</sup>I-NGF were separated using a Beckman Airfuge. 75-μl samples (containing 75 μg of protein) were layered onto 50 μl of PBS containing 0.1% serum albumin and 10% sucrose in Airfuge tubes. Tubes were centrifuged at 85,000 × g for 5 min and frozen. Pellets were removed and counted as described previously. Nonspecific binding was less than 40% of total, and the efficiency of binding to membranes compared to cells was approximately 25%.

RESULTS

Plasma membrane receptors are usually glycoproteins and consequently provide binding sites for lectins exhibiting appropriate carbohydrate specificity. Lectins, when bound to hormone receptors, often inhibit binding of the specific ligand via direct steric interference or by inducing a conformational change in the hormone-binding site which significantly lowers the equilibrium binding affinity of the receptor for the hormone. This phenomenon has been reported for epidermal growth factor (17), prolactin (18), and more interestingly insulin (19) in which lectin bound to insulin receptor elicits a characteristic insulin-like cellular response. Lectins can, therefore, be utilized as tools to probe the composition of carbohydrate moieties on receptor glycoproteins and more importantly can be employed to investigate the effect of receptor perturbation on receptor function.

The effect of various lectins on NGF binding was investigated by incubating PC12 cells with a battery of lectins followed by quantitation of <sup>125</sup>I-NGF binding. As shown in Table I, only one lectin, WGA, significantly inhibited NGF binding. The magnitude of inhibition is dependent on WGA concentration during pretreatment and, as illustrated by Fig. 4A, NGF binding is inhibited 50% at a WGA concentration of 50 ng/ml. This dose-dependent relationship is similar to the lectin-mediated inhibition of epidermal growth factor and prolactin binding cited previously. Another similarity shared with the previous examples (17, 18) is the observation that a fraction of receptors is seemingly resistant to lectin-induced inhibition of ligand binding regardless of the concentration of lectin utilized. Perhaps these NGF receptors lack carbohydrate moieties or they are inaccessible for lectin binding.

With the expectation that WGA might preferentially inhibit binding to either Slow or Fast receptors, we determined the effect of WGA on the proportion of NGF bound to these two receptor classes. The amount of NGF bound to Slow receptors was measured by the standard protocol which involves saturating receptors with <sup>125</sup>I-NGF and then incubating cells with unlabeled NGF to permit complete dissociation of

<table>
<thead>
<tr>
<th>Lectin</th>
<th>Sugar specificity</th>
<th>NGF bound (µg/ml)</th>
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<tbody>
<tr>
<td>None/control</td>
<td></td>
<td>100% ± 1.4</td>
</tr>
<tr>
<td>Concanavalin A</td>
<td>Mannose/glucose</td>
<td>98 ± 7.9</td>
</tr>
<tr>
<td>Dolichoes</td>
<td>GalNac</td>
<td>90 ± 5.0</td>
</tr>
<tr>
<td>Lotus</td>
<td>Fucose</td>
<td>100 ± 12</td>
</tr>
<tr>
<td>Peanut</td>
<td>Gal</td>
<td>100 ± 9.9</td>
</tr>
<tr>
<td>Soybean</td>
<td>GalNac</td>
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</tr>
<tr>
<td>Ulex</td>
<td>Fucose</td>
<td>102 ± 6.9</td>
</tr>
<tr>
<td>WGA</td>
<td>GluNac</td>
<td>39 ± 8.6</td>
</tr>
<tr>
<td>Diphtheria toxina</td>
<td>GluNac</td>
<td>104 ± 5.7</td>
</tr>
</tbody>
</table>

Concentration of diphtheria toxin during preincubation was 50 µg/ml.
$^{125}$I-NGF from Fast receptors without significant loss of $^{125}$I-NGF from Slow receptors. WGA unexpectedly caused a dramatic increase in the proportion of NGF bound to Slow receptors relative to the NGF bound to Fast receptors. (Fig. 1A). This effect cannot be due simply to preferential inhibition of NGF binding to Fast receptors. In fact, it does not appear that preferential inhibition of Fast receptors is a factor. Table II demonstrates that when PC12 cells were treated with sufficient trypsin to inactivate all Fast receptors, binding of NGF to the remaining Slow receptors was effectively inhibited by WGA.

The paradoxical observation that WGA increases the absolute amount of NGF bound to Slow receptors even while inhibiting NGF binding to Slow receptors apparently results from the fact that a WGA-induced conversion of Fast to Slow receptors occurs, thus compensating for WGA inhibition of NGF binding to pre-existing Slow receptors. This phenomenon is more obvious in experiments in which WGA was added to PC12 cells after NGF binding rather than prior to NGF binding. Fig. 1B illustrates that under these conditions, WGA causes an increase in the amount of slowly dissociating NGF and a corresponding decrease in rapidly dissociating bound NGF while causing little or no inhibition of total NGF binding. This process appears to involve a WGA-induced conversion of Fast to Slow receptors. Additional support for this hypothesis will be presented. From Fig. 1A and B, it can be seen that the WGA concentration required to convert Fast to Slow receptors via WGA addition following NGF binding is

![Fig. 1. Effect of WGA on NGF binding. A, PC12 cells were treated with WGA at various concentrations for 30 min at 37°C and then incubated for an additional 30 min following the addition of 20 ng/ml of $^{125}$I-NGF. Total specific binding (O) and the proportion of specifically bound NGF which was slowly dissociating (0) were measured as described under "Methods." Data was corrected for nonspecific binding. Total specific binding in the absence of WGA was 11,800 cpm per 10^5 cells. B, PC12 cells were first incubated with 20 ng/ml of $^{125}$I-NGF for 30 min at 37°C and then, following the addition of WGA at the concentration indicated, cells were incubated for an additional 30 min. Total bound (O) and slowly dissociating (0) NGF was measured as in A. Total specific binding in the absence of WGA was 35,800 cpm per 10^5 cells.](http://www.jbc.org/)

![Fig. 2. Effect of temperature on the kinetics of WGA-induced NGF receptor conversion. PC12 cells were incubated with $^{125}$I-NGF (20 ng/ml) at 37°C for 30 min, and 100 ng/ml of WGA were then added. Following incubation at either 37°C (O) or 4°C (C) for the time indicated, aliquots of cells were removed and the proportion of slowly dissociating bound NGF was measured as described under "Methods." Total specific binding prior to WGA addition was 3000 cpm per 10^5 cells at 0°C and was 8900 cpm per 10^5 cells at 37°C.](http://www.jbc.org/)

![Fig. 3. Effect of WGA on the affinity of NGF for its receptor. PC12 cells were incubated for 30 min at 37°C with $^{125}$I-NGF at the concentrations indicated, at which time total NGF bound to cells was measured as described under "Methods." A, total $^{125}$I-NGF associated to cells with no prior treatment (O) and total bound to cells incubated with WGA (100 ng/ml) for 30 min at 37°C prior to association with $^{125}$I-NGF (C). B, data replotted according to Scatchard. Assuming that NGF exists in solution and binds to receptor as a 26,000-dalton dimer, the Scatchard plot extrapolation of 300 pg of NGF per 10^5 cells corresponds to 70,000 receptors per cell. (26 ng/ml = 1 nM NGF).](http://www.jbc.org/)
10-fold lower than the WGA concentration required to inhibit NGF binding when WGA is added prior to NGF.

The kinetics of WGA-induced conversion is rapid and as depicted in Fig. 2 proceeds at a similar rate at both 37 and 4°C. The apparent lack of temperature dependence suggests that extensive topographical movement of membrane components or metabolic processes is not involved. This conclusion is further supported by the inability of sodium azide, in the absence of glucose, to block WGA-induced conversion of NGF receptors. In particular it is clear that the WGA-induced increase in slowly dissociating NGF is not merely due to increased endocytic cellular uptake of NGF since this uptake, for NGF as well as for many other peptide hormones, is efficiently blocked by low temperature or inhibition of production of metabolic energy (20, 21).

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![Fig. 4. Reversibility of WGA-induced NGF receptor conversion.](image_url)

**Fig. 4.** Reversibility of WGA-induced NGF receptor conversion. Cells were incubated with [125I]-NGF (25 ng/ml) for 30 min at 37°C. WGA (100 μg/ml) was added, the cell suspension was incubated for 30 min and then 100 mM GlcNAc was added. Aliquots of cell suspension were removed at the times indicated and total bound NGF was measured as described under "Methods." Labeled NGF bound to Slow receptors was measured by gently pelleting the cell aliquot, resuspending the cell pellet in ice-cold binding buffer containing 10 μg/ml of unlabeled NGF, and incubating at 0°C for 30 min, after which time cells were pelleted in the microfuge for assay of bound [125I]-NGF as described under "Methods." The proportion of Slow receptors exhibited by cells not treated with lectin is indicated by the arrow. Total specific binding prior to addition of GlcNAc was 21,400 cpm per 10⁶ cells.

**Fig. 5.** Effect of WGA on dissociation and association kinetics of PC12 NGF receptors. A, NGF dissociation kinetics was determined by associating [125I]-NGF (20 ng/ml) to PC12 cells for 30 min at 37°C, adding 10 μg/ml of unlabeled NGF either directly (●) or following a 30-min incubation with 50 μg/ml of WGA (○), and removing aliquots of cell suspension at the times indicated for measurement of bound [125I]-NGF as described under "Methods." Total specific binding was 8100 cpm per 10⁶ cells at 37°C and was 7700 cpm per 10⁶ cells at 0°C. B, NGF association kinetics was determined by incubating cells with [125I]-NGF (20 ng/ml, 4°C) and removing aliquots of cell suspension at the times indicated for measurement of total bound NGF (●) and NGF bound to Slow receptors (■) as described under "Methods." The effect of WGA on NGF association (○) was determined by incubating cells with 100 μg/ml of lectin for 30 min at 37°C prior to addition of [125I]-NGF.

The nature of WGA inhibition of NGF binding was characterized by Scatchard analysis (22) of equilibrium binding data. This analysis, illustrated by Fig. 3, shows that inhibition of NGF binding by 100 μg/ml of WGA is primarily due to a 3-fold decrease in NGF affinity (4.2 × 10⁻¹⁰ M = 12 ng/ml versus 1.4 × 10⁻⁹ M = 36 ng/ml) with a small and possibly insignificant reduction in total NGF receptor number from 300 pg/10⁶ cells (70,000 sites per cell) to 240 pg/10⁶ cells (56,000 sites per cell). Apparent equilibrium constants were calculated from the lines drawn in Fig. 3B, using the value 26,000 for the molecular weight of NGF. A similar decrease in NGF receptor affinity was recently reported by Costrini and Kogan (23) who observed a 6-fold reduction of rabbit superior cervical ganglia receptor affinity for NGF. This reduction of receptor affinity was induced by WGA and concanavalin A.

Sugar recognition evidently plays an important role in the mechanism of WGA action since the presence of 100 mM GlcNAc during pretreatment of PC12 cells with 100 μg/ml of WGA results in a reduction of NGF binding by only 20% compared to 80% by WGA alone. Although diphtheria toxin also exhibits GlcNAc binding specificity (24), pretreatment of PC12 cells with this toxin exerts no effect upon subsequent NGF binding. The conversion of Fast NGF receptors to Slow by WGA is also dependent on GlcNAc recognition since this process is completely blocked by the presence of 100 mM GlcNAc. Not only does GlcNAc prevent receptor conversion, but addition of this sugar to cells which have already undergone receptor conversion reverses the effects of WGA and these cells regain their initial distribution of NGF receptors between Fast and Slow forms. The reversibility is sugar specific, occurs at 4°C, and as illustrated by Fig. 4 is nearly complete in 30 min at 37°C. The rapid reversibility of this process constitutes additional evidence that internalization of NGF does not significantly contribute to the increase in slowly dissociating NGF induced by WGA. A minor component (about 15%) of converted receptors apparently fail to revert to Fast kinetics following GlcNAc addition, and it is possible that this small subtraction represents receptor internalization. More probably, however, it merely reflects incomplete competition by the GlcNAc concentration employed. The reduction of NGF binding by WGA also apparently does not involve WGA-induced receptor internalization and/or degra-
The effect of WGA on NGF receptor sensitivity to trypsin was investigated by treating PC12 cells with 125I-NGF (20 ng/ml), WGA (100 ng/ml) and trypsin (20 ng/ml) in the sequences depicted below. The duration of each sequential incubation was 30 min at 37 °C. Cell-associated NGF was measured as described under "Methods." All data is corrected for nonspecific binding and is expressed as counts per min bound per 10⁶ cells.

<table>
<thead>
<tr>
<th>Treatment of PC12 cells</th>
<th>Total cpm bound</th>
<th>% trypsin resistant</th>
</tr>
</thead>
<tbody>
<tr>
<td>NGF</td>
<td>7489*</td>
<td></td>
</tr>
<tr>
<td>NGF → trypsin</td>
<td>3624</td>
<td>35% ± 2.9</td>
</tr>
<tr>
<td>NGF → WGA</td>
<td>8224*</td>
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<td>NGF → WGA → trypsin</td>
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<td>93% ± 5.1</td>
</tr>
<tr>
<td>Trypsin → NGF</td>
<td>974</td>
<td>13% ± 0.9</td>
</tr>
<tr>
<td>WGA → trypsin → NGF</td>
<td>1124</td>
<td>15% ± 3.3</td>
</tr>
</tbody>
</table>

A. Schechter, unpublished data.

A number of experiments were performed to determine to what extent the WGA-induced Slow receptor state is similar or functionally equivalent to pre-existing Slow receptors. First, the rate of 125I-NGF dissociation from WGA-treated cells was measured following addition of a large excess of unlabeled NGF, and as shown in Fig. 5A the rate of dissociation from WGA-treated cells is similar to the rate from the Slow receptor population on untreated cells. The two populations of receptors are evident in each case, but the Slow component on the WGA-treated cells is 3-fold more abundant compared to the Slow component on the untreated cells. Another common feature of these two populations of Slow receptors is that NGF does not dissociate from them at a measurable rate at 4 °C. Association kinetics of 125I-NGF to PC12 cells previously treated with WGA was measured and, as illustrated in Fig. 5B, the majority of receptors bind NGF at a rate similar to Fast rather than Slow receptors.

A distinctive feature of Slow receptors is their relative resistance (compared to Fast receptors) to inactivation by trypsin. This difference is observed when receptors are inactivated both prior to NGF addition (14) or when NGF is already bound (15), although in the latter case the presence of NGF somewhat lowers the rate of inactivation of both receptor classes (see Table III). Table III demonstrates that the Slow receptor state induced by incubation with NGF followed by addition of WGA is completely resistant to inactivation by trypsin under conditions sufficient to inactivate essentially all Fast receptors. However, WGA alone does not confer trypsin resistance to NGF receptors. This result, like the observation that 125I-NGF binds to WGA-treated cells with the kinetics of Fast rather than Slow receptors, indicates that lectin-induced conversion depends, at least in part, on NGF occupancy of receptors. Increased trypsin resistance was not observed when cells were incubated with NGF and then concanavalin A. Thus the effect of WGA is apparently specific and not merely due to an indirect effect of extensive interaction of the lectin with the cell surface.

It has been surmised that pre-existing Slow NGF receptors may be associated with cytoskeletal elements based on the observation that NGF bound to Slow receptors is preferentially retained on structures obtained by Triton X-100 extraction of cells using buffers chosen to stabilize cytoskeletal structures (25). As shown in Table IV, the WGA-induced Slow receptors, like the pre-existing population, are resistant to solubilization by Triton detergent under these conditions. Investigations of NGF binding to purified PC12 plasma membrane preparations suggest that cytoskeleton or some other cytoplasmic component is required for formation and maintenance of the Slow receptor state. Table V indicates that virtually all of NGF bound to PC12 membranes dissociates with the kinetic rate constant of Fast receptors. WGA neither inhibits NGF binding to receptors on membrane preparations nor causes the conversion of Fast to Slow receptors.

Recently we have begun to investigate the effects of WGA on NGF receptors on human melanoma A875 cells. As shown in Table V, A875 cells have a smaller proportion of NGF receptors with dissociation kinetics comparable to PC12 Slow receptors. The effects of WGA on A875 NGF receptors are very much like those on PC12 NGF receptors. As seen in Table V, incubation with 125I-NGF followed by WGA produces a receptor state from which NGF dissociates slowly, while incubation with WGA prior to NGF addition leads to inhibition of NGF binding in addition to formation of Slow receptors. The rates of dissociation of NGF from Fast and Slow forms of A875 NGF receptors are very similar to those observed for PC12 receptors (data not shown).

One notable difference exists between PC12 and A875 cells. While WGA does not noticeably affect the behavior of NGF receptors on membrane preparations of PC12 cells, WGA induces NGF receptor conversion in membrane preparations from A875 cells in a manner very much like that in whole
cells. As shown in Table V, WGA causes Fast to Slow receptor conversion, when lectin is added following incubation with 125I-NGF. Another effect of WGA, not seen on whole cells, is a large increase in amount of NGF bound. It is possible that this apparent increase is an artifact of the assay method. The relatively slower rate of sedimentation of membrane vesicles compared to whole cells may permit significant dissociation of NGF from Fast receptors during the centrifugational separation of free from bound NGF. The ability of WGA to slow the rate of dissociation of NGF from receptors would reduce the extent of this underestimation of amount bound and would lead to an apparent increase in the amount of bound NGF. In any case, A875 membranes appear to differ from PC12 membranes in retaining the ability of NGF receptors to be converted from the Fast to Slow form in the presence of WGA. Since it is very unlikely that endocytotic internalization of receptors would occur to any significant extent in a purified membrane preparation, this constitutes additional evidence that Fast to Slow receptor conversion does not involve endocytosis.

The possible involvement of interactions with microtubules or microfilaments in the generation of Slow receptor characteristics was tested using drugs which interfere with the function of these cytoskeletal elements. Pretreatment of PC12 cells with colchicine (2 μg/ml) or cytochalasin B (50 μg/ml) or both simultaneously did not affect the proportion of pre-existing Slow receptors and failed to abrogate the effect of WGA-induced conversion of Fast to Slow receptors. (In control experiments, treatment of HeLa cells with these drugs at the stated concentrations caused extensive morphological changes.) This suggests that the effect of WGA does not require active modulation of microtubules or microfilaments.

**DISCUSSION**

In the majority of systems characterized to date, NGF receptors exhibit heterogeneity with respect to equilibrium and/or kinetic binding constants. Chick sensory ganglia cells (23), and PC12 cells (13, 14, 26) all show NGF receptor heterogeneity. Although the basis of this heterogeneity is unknown, similar observations have been reported for a variety of other peptide hormone systems including receptors for insulin (27), epidermal growth factor (28) and endogenous opiates (29). Although the question is not without controversy, in the case of these latter receptors (27–29) heterogeneity is apparently dependent on ligand binding, i.e. hormone binding to receptors induces heterogeneity in an initially homogeneous system. The basis for the heterogeneity of NGF receptors has been a perplexing question to answer. For receptors of sensory and sympathetic nerve cells, some investigators have concluded that heterogeneity exists prior to NGF exposure (16) while others have indicated that NGF binding induces the heterogeneity (9); still others suggest that receptor heterogeneity is the combined result of pre-existing and NGF-induced receptor conversion (12). For PC12 cells, a report by Landreth and Shooter (13) concluded that NGF-induced receptor conversion results in NGF receptor heterogeneity, whereas a report from this laboratory concluded that receptor heterogeneity exists prior to NGF binding (14).

While we continue to believe that the differential sensitivity to trypsin inactivation of Fast and Slow NGF receptors (in the absence of NGF) compellingly demonstrates that these receptor classes exist prior to NGF exposure, we now have extended these findings by showing that simultaneous exposure of PC12 cells to WGA and NGF converts Fast receptors to a form which is nearly indistinguishable from pre-existing Slow receptors, not only in rate of dissociation of bound NGF, but also in resistance to extraction by Triton X-100 and to inactivation by trypsin. Thus, although NGF apparently does not normally cause conversion of Fast to Slow receptors, in the presence of WGA this conversion can take place. This suggests that Fast and Slow receptors represent interconvertible states of a single protein rather than different proteins.

The addition of WGA alone does not induce Fast receptors to exhibit Slow behavior since WGA does not confer resistance to trypsin inactivation of receptor unless NGF is also present. This conclusion is also supported by the fact that NGF binds to WGA-treated cells with the kinetic rate constant of Fast rather than Slow receptors. Thus, synergistic action between WGA and NGF is apparently required to convert Fast to Slow receptors. A provocative feature of this interaction is that a different end state results depending on whether NGF is added before or after WGA. Addition of WGA followed by NGF leads to receptor conversion along with reduced NGF binding, while addition of NGF followed by WGA yields receptor conversion without reduced NGF binding (Fig. 1). Another manifestation of this phenomenon is that the WGA concentration required to induce Fast to Slow receptor conversion is greater when WGA is added before NGF compared to the opposite order of addition.

In Fig. 6 we present one plausible model, for the sole purpose of illustrating how the order of addition of NGF and WGA might determine which of two noninterconvertible end states is formed. The model assumes that tetravalent WGA cross-links NGF receptors causing formation of small receptor clusters and postulates that NGF bound to receptor masks some subset of lectin-binding sites, so that different modes of receptor clustering result depending on whether receptors do or do not have bound NGF when WGA is added. Since receptor-lectin interactions in such clusters would be multivalent, as depicted in Fig. 6, the interconversion between the two postulated forms of clustered receptor might be very slow, while the interaction of NGF with the various forms of the receptor would be readily reversible. For illustrative purposes the model depicts formation of NGF receptor dimers via lectin cross-linking. However, other plausible alternatives include lectin cross-linking-induced formation of larger NGF receptor aggregates, complexes of NGF receptors with other membrane components, or simply a change in NGF receptor conformation caused by multiple attachment of multivalent lectin molecules. In any case, the proposed steric interference between bound WGA and NGF in only one of two possible configurations would account for the observation that receptors show reduced affinity for NGF when WGA is added prior to NGF, but not when the order of addition is reversed.
The fact that the end state is dependent on order of addition of components necessarily implies that the end states do not represent true equilibria and, therefore, Scatchard analysis of data must be viewed with caution. Equilibrium analysis will be appropriate only if the rates of NGF equilibration with the two end states depicted in Fig. 1 are rapid compared to the rates of interconversion between the various receptor states. However, to the extent that an equilibrium analysis is meaningful, it appears that WGA inhibition to NGF binding involves primarily a reduction of NGF affinity for receptors, with relatively little change in number of available receptors. An apparent paradox is presented by the fact that the presence of WGA causes NGF to dissociate more slowly from receptors (Fig. 5) without affecting the rate of association of NGF with receptors (Fig. 2) and, therefore, the dissociation equilibrium constant, calculated as the ratio of kinetic constants, should be decreased yet the Scatchard analysis of "equilibrium" binding data (Fig. 3) indicates that the equilibrium constant is increased. The explanation for this discrepancy lies in the fact that the kinetic association and equilibrium experiments involved incubation with WGA before NGF, while the kinetic dissociation experiments involved addition of NGF before WGA. Since different end states clearly result, dependent on order of addition of WGA and NGF (as depicted in the model) the above calculation of an equilibrium constant from kinetic constants is invalid, because it involves kinetic "on" and "off" constants from two distinct reactions.

Equilibrium analysis suggests that the lower reaction pathway depicted in the model (Fig. 6) leads to a receptor state with decreased affinity for NGF while kinetic analysis suggests that the receptor state which results from the upper reaction pathway has an increased affinity for NGF. Thus it is possible that the lack of change in the amount of NGF bound when WGA is added after NGF (Fig. 1) is due to the cancelling effects of a portion of receptors following the upper reaction pathway leading to increased NGF binding and a portion of receptors following the lower reaction pathway leading to reduced NGF binding.

The mechanism whereby WGA induces the interconversion of NGF receptors is unclear, but some important aspects of the process have been defined. Specific sugar recognition is apparently involved, since both inhibition of NGF binding and Fast to Slow receptor conversion by WGA are rapidly reversed by addition of GlcNAc. Epifluorescent microscopy of PC12 cells treated with fluorescein-conjugated WGA has shown that bound lectin remains dispersed over the cell at 4 °C but rapidly forms clusters at 37 °C. Since it has been reported that NGF receptors cluster as the result of NGF binding (21), it is tempting to speculate that these two dynamic events may be involved in the WGA-induced conversion of NGF receptors. However, if receptor clustering or other topographical redistribution of plasma membrane components is involved, it must be extremely limited in nature, since WGA-induced receptor conversion occurs with nearly identical rates at 4 and 37 °C, while receptor clustering and other processes requiring membrane fluidity are generally strongly inhibited by reduction of temperature. However, small receptor clusters can form in response to ligand binding, even at 0 °C as shown for α2-macroglobulin receptor by Willingham et al. (30). Receptor conversion is apparently metabolically passive in nature since conversion occurs in the presence of sodium azide in medium without glucose. Active participation of microfilaments and microtubules are probably not involved since receptor conversion is unaffected by pre-treatment of cells with cytochalasin B, colchicine, or both together. It seems highly unlikely that receptor internalization plays a significant role in the observed effects of WGA since 1) the rate of WGA-induced receptor conversion is unaffected by lowered temperatures or inhibitors of metabolic energy production, both of which generally block endocytosis, 2) the effects of WGA are rapidly reversed by GlcNAc, and 3) a similar phenomenon is seen in isolated A875 cell membranes.

We have discussed models involving a conformational change in receptor induced by WGA binding to NGF receptors, lectin-induced microclustering of NGF receptors, or lectin-induced interaction of adjacent membrane glycoproteins with NGF receptor. The possibility that the effect of WGA is communicated indirectly by binding of WGA to membrane sites other than NGF receptor cannot be excluded. However, it is very probable that the effect involves a direct interaction with the NGF receptor since recent results in this laboratory demonstrate that the NGF receptor extracted from PC12 or A875 cells with Triton X-100 detergent binds specifically to WGA-Sepharose affinity columns (31).

A somewhat different model to explain the action of WGA is the reduction of lateral mobility of membrane components induced by lectin binding. It has recently been shown that NGF receptors are initially dispersed over the cell surface but form clusters, presumably over coated pits, when occupied (21). Elimination of this lateral mobility, in a similar fashion to that by which concanavalin A reduces mobility of membrane components (32, 33), may impose a steric or conformational restriction upon the receptor which results in the characteristic Slow receptor phenotype. A similar restriction may be imposed upon a small percentage of receptors in the absence of WGA, and these would constitute the pre-existing Slow receptor population. Any model which is proposed to account for the effects of WGA on NGF receptor must be capable of explaining why different end states result depending on the order of addition of NGF and WGA. It would be premature to settle on any particular model in view of the fragmentary nature of our knowledge. The proposed steric interference between bound WGA and NGF in only one of the two possible modes of WGA cross-linked receptor clustering would account for the observation that receptors show normal affinity for NGF when WGA is added prior to NGF, but not when the order of addition is reversed.

The extensive similarities between the properties of the pre-existing subpopulation of Slow receptors and the state produced by treatment with NGF followed by WGA strongly suggest that these receptor states are related by related mechanisms and hence the stabilization by WGA of the Slow receptor state should facilitate characterization of the molecular interactions involved. In particular it remains to be shown whether the preferential association of the Slow class of receptors with Triton detergent-insoluble "cytoskeleton" reflects 1) a direct interaction of receptors with cytoskeletal proteins, 2) a specific interaction of receptors with membrane proteins which are themselves linked to cytoskeletal proteins, or 3) an interaction in which cytoskeletal proteins have no direct bearing. In this regard, the fact that the WGA-induced receptor conversion is lost in purified PC12 membrane preparations but is retained in A875 membrane preparations suggests that a comparison of these two systems will be useful in the future for investigating the mechanism of generation of Slow receptors. Do A875 membrane preparations retain a greater amount of cytoskeletal material? Can exogenously added cytoskeleton proteins or other A875 proteins restore the effect of WGA on PC12 membrane NGF receptors? Questions such as these will be addressed in the future.

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2 P. Grob, unpublished data.
Since this work was completed two reports have been published which describe similar results. Vale and Shooter (34) examined the effects of WGA on NGF receptors on intact PC12 cells with results and interpretations very similar to our own. They also found that WGA caused a similar NGF receptor conversion on chick sensory nerve cells. The effects of WGA on NGF binding to A875 cell membranes containing NGF receptors, as well as detergent solubilized receptors and receptors reconstituted into phosphatidy Choline vesicles, has been investigated by Buxser et al. (35). Although these investigators, in contrast to ourselves, observed little WGA-induced inhibition of NGF binding to A875 cell NGF receptors, they did observe the WGA-induced change in NGF dissociation kinetics and reduced sensitivity to trypsin, in agreement with our results. Thus, taking our own results together with those of these two other groups, it appears that the ability of WGA to convert NGF receptors to a form from which NGF dissociates more slowly is a very general phenomenon, existing on chick sensory neurons, rat (adrenal) pheochromocytoma cells, and human melanoma cells. The evolutionary conservation of this effect suggests that it reflects some functionally important aspect of receptor function.

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Modification of nerve growth factor receptor properties by wheat germ agglutinin.

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