

# Nerve Growth Factor Nonresponsive Pheochromocytoma Cells: Altered Internalization Results in Signaling Dysfunction

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**Abstract.** Variant rat pheochromocytoma (PC12) cells which fail to respond to nerve growth factor (NGF) (PC12nnr5) (Green, S. H., R. E. Rydel, J. L. Conolly, and L. A. Greene. 1986. *J. Cell Biol.* 102:830–843) bind NGF at both high and low affinity sites. Although still undefined at the molecular level, these have been referred to as type I (high) and type II (low) receptors. They are apparently composed of two membrane-bound proteins, p75 and the protooncogene *trk*, both of which bind NGF, and apparently contribute singularly or in concert to the two observed affinities, and to the promotion of the NGF effects. In native PC12 cells, only the high affinity receptors are apparently capable of mediating internalization and degradation. PC12nnr5 cells also display type I bind-

ing, but the subsequent internalization is not the same fashion as in the parental cell line, nor is it subjected to lysosomal degradation. Rather it is initially sequestered during the first 15 min, and is eventually released intact into the medium. In contrast, EGF is bound, internalized, and degraded by PC12nnr5 cells, albeit less efficiently than in the parent cells. These observations argue that the defect(s) preventing the PC12nnr5 variants from responding to NGF prevents competent internalization, which in the case of NGF, may be required for the full expression of activity. The absence of *trk*, as one alteration in PC12nnr5 cells (Loeb, D. M., J. Maragos, D. Martin-Zanca, M. V. Chao, L. F. Parada, and L. A. Greene. 1991. *Cell.* 66:961–966), is consistent with this conclusion.

THE rat pheochromocytoma line PC12 (Greene and Tischler, 1976) is a widely used cultured cell system for the study of neurotrophic factor-induced differentiation. Upon exposure to nerve growth factor (NGF),<sup>1</sup> for example, PC12 cells cease cell division, extend neurites, and produce the many proteins needed to become functioning neurons (for review see Greene and Tischler, 1982). These reversible effects are mediated by the binding of NGF to specific receptors on the plasma membrane which results in the production of a variety of intracellular signals that combine to produce the differentiated phenotype (Altin and Bradshaw, 1992). An important aspect of these and other NGF-responsive cells is the existence of at least two distinct classes of NGF receptors that are distinguished by their affinity (Sutter et al., 1979; Schechter and Bothwell, 1981; Hosang and Shooter, 1985). Binding experiments generally reveal a relatively small number of type I (high affinity,  $K_d \sim 0.4$  nM) NGF receptors and a larger number of type II (low affinity,  $K_d \sim$  nM) receptors (Sutter et al., 1979; Schechter and Bothwell, 1981; Bernd and Greene, 1984). These binding constants differ primarily by the apparent off-rate and the corresponding receptors are sometimes referred to as "slow" (type I) and "fast" (type II). The two receptor forms apparently

arise from the internalization of occupied surface receptors (Eveleth and Bradshaw, 1988; Buxser et al., 1990).

The molecular definition of the NGF receptors in PC12 cells (or responsive neurons) remains obscure. Initial chemical characterization of NGF receptors from responsive cells using hydrodynamic (Costrini et al., 1979) and cross-linking (Massague et al., 1981) methodologies suggested values of 130–140 kD for NGF receptors of sympathetic neurons and somewhat higher values (150–160 kD) for PC12 cells (Massague et al., 1982). However, similar approaches with different reagents applied to nonresponsive human melanoma cells identified only an 80-kD species (as well as a 200-kD form in lesser amounts, thought to be a dimer) (Grob et al., 1983; Puma et al., 1983). A similar 80-kD species was eventually observed in PC12 cells. Subsequent cloning experiments have provided sequences for the low molecular weight NGF receptor from both human melanoma (Johnson et al., 1986) and PC12 (Radeke et al., 1987) cells. Both sequences predict proteins of <50 kD. They are assumed, along with both N- and O-linked carbohydrates (and attached ligand), to make up the 75–80-kD species seen in cross-linking experiments. This form of the NGF receptor apparently provides cells with the capability of type II binding and is now generally referred to as LNGFR (or p75); the higher molecular weight forms arise either from association (and cross-linking) of this receptor with an accessory protein(s), or represent other larger NGF receptors not related to the low

1. *Abbreviations used in this paper:* bFGF, basic fibroblast growth factor; NGF, nerve growth factor; PC12, rat clonal pheochromocytoma cells; PC12nnr5, NGF nonresponsive variant of PC12 cells.

molecular weight form (Kouchalakos and Bradshaw, 1986). The recent identification of the protooncogene *trk* as an NGF-binding entity in some NGF-responsive cells suggests that the latter model is more likely, at least in the majority of cases (Klein et al., 1991; Kaplan et al., 1991; Hempstead et al., 1991). Only the high molecular weight forms can generate type I (high affinity) binding and biological activity (Bernd and Greene, 1984).

Green et al. (1986), using ethyl methane sulfonate mutagenesis, generated PC12 variants which did not respond to NGF. Several cell lines, designated nnr (NGF nonresponsive), were identified which displayed similar phenotypes: failure to extend neurites in response to NGF, apparent lack of high affinity NGF binding sites (as measured by equilibrium binding and Scatchard analysis) but normal low affinity sites, and a failure to internalize NGF. These cells are capable of internalizing EGF (Green et al., 1986) and the PC12nnr5 line retains the ability to extend neurites in response to cAMP (Green et al., 1986) and *v-src* (Eveleth et al., 1989) as has been reported for PC12 cells (Alema et al., 1985).

In this report, we have examined the binding and processing of NGF by PC12nnr5 cells and find that (a) they do show high affinity binding (under nonequilibrium conditions); (b) they can sequester and/or internalize NGF in a limited fashion; and (c) they cannot further process or degrade the hormone. Thus, high affinity binding cannot be strictly tied to either biological response or a particular form of the receptor. These findings also further connect competent signal transduction with an endocytotic pathway that can lead to efficient lysosomal degradation. A defect in this process may render cells incapable of responding to a specific ligand.

## Materials and Methods

### Cells and Growth Factors

PC12 and PC12nnr5 rat pheochromocytoma cells were kindly provided by Dr. Lloyd Greene, New York University, and were grown on collagen-coated dishes in high glucose DME supplemented with 10% heat-inactivated horse serum and 5% FCS (Irvine Scientific, Santa Ana, CA) (complete medium). NGF was prepared from male mouse submaxillary glands (Pel-Freez Biologicals, Rogers, AR) by the method of Bocchini and Angeletti (1969). Iodination of NGF was performed as described by Sutter et al. (1979). The EGF from male mouse submaxillary glands used in these studies was prepared by the method of Savage and Cohen (1972) and iodinated as described by Wiley and Cunningham (1982). These reagents were generously provided by Michael Blaber and Kathy Cavanaugh (University of California, Irvine), respectively.

### Binding and Internalization

For the measurement of NGF binding, cells were plated at high density in 35-mm dishes (Linbro) coated with collagen (Vitrogen) and allowed to grow 48 h before assay. All cells were fed within 24 h. Total binding was measured by incubating the cells with  $^{125}\text{I}$ -NGF in complete medium for 15 min at 37°C, rinsing twice in ice-cold DME, and solubilizing in 1 M NaOH overnight. Since the incubation times in these experiments are short enough that equilibrium between bound and free ligand may not have been reached and since ligand internalization by the two cell types during incubation is different, the values obtained from the Scatchard analysis cannot be considered accurate estimations of the binding constants of the NGF sites on these cells but rather should only be used to compare binding on the two cell types. The derived binding constant and number of binding sites per cell for the PC12 cells is, nonetheless, similar to those previously reported (Bernd and Greene, 1984).

Determination of the amount of internal and external labeled hormone

was performed by the acid washing technique of Haigler et al. (1980), as described by Bernd and Greene (1984). Cells were exposed to 0.2 nM  $^{125}\text{I}$ -NGF in complete medium for various lengths of time, quickly rinsed twice with ice-cold DME, and placed on ice; and the surface-bound  $^{125}\text{I}$ -NGF was removed in 0.2 M acetic acid/0.5 M NaCl for 5 min. After rinsing to remove traces of surface-bound label, the radioactivity remaining (solubilized in 1 M NaOH overnight) was designated as internalized. Cell number on plates was quantitated by counting the plates after the acid washing step.

Assays of total specific NGF binding as well as specific binding to type I and type II receptors were performed as described by Schecter and Bothwell (1981). PC12 cells ( $10^4$ – $10^5$  per tube) were mixed with labeled NGF (10 ng/ml) in a total volume of 400  $\mu\text{l}$  and incubated in suspension at 37°C for 30 min. 100- $\mu\text{l}$  aliquots of the suspension were layered onto 300  $\mu\text{l}$  of 10% sucrose in microfuge tubes and centrifuged for 15 s at 10,000 g. The tubes were frozen and the tips containing the cells were cut off and counted in a gamma spectrometer (model 4000, Beckman Instrs., Inc., Fullerton, CA). For each data point nonspecific binding (30–60% of total) was determined in the presence of an excess (5  $\mu\text{g}/\text{ml}$ ) of unlabeled NGF. Specific binding was considered to be the amount of total binding less the amount bound in the presence of excess cold NGF (nonspecific binding). All error estimates reflect the error of the total bound ligand; the error in nonspecific binding was not estimated.

### Degradation

Analysis of the degradation of  $^{125}\text{I}$ -NGF to iodotyrosine was performed according to the method of Wiley and Cunningham (1982). Cells in suspension in complete medium were incubated (loaded) with 0.2 nM  $^{125}\text{I}$ -NGF for 10 or 30 min. Unbound NGF was removed by washing the cells twice in warm complete medium. The loaded cells were then incubated in complete medium at 37°C in the presence of 0.2 nM unlabeled NGF for various amounts of time. At the end of the incubation, aliquots of cells and medium were mixed with 0.1 vol of loading buffer, 100 mM CHAPS (Pierce Chemical Co., Rockford, IL), 5% glycerol, 20 mM EDTA, 0.01% bromophenol blue, 20 mM PMSF made up in DME. This mixture was incubated at least 20 min on ice, centrifuged 5 min at 12,000 g to remove cellular debris, and layered on acrylamide tube gels. After electrophoresis, the gels were frozen and sectioned into 2-mm slices. Under the buffer conditions used in these gels, NGF will not migrate into the resolving gel and thus not all of the radioactivity loaded onto the gels was recovered in the slices. As a control for the degradation of the NGF by cell lysates, an aliquot of unloaded cells was added to sample buffer, followed by  $^{125}\text{I}$ -NGF; the mixture was incubated for 90 min and electrophoresed as the other samples. No radioactivity was found migrating with the dye front in these controls.

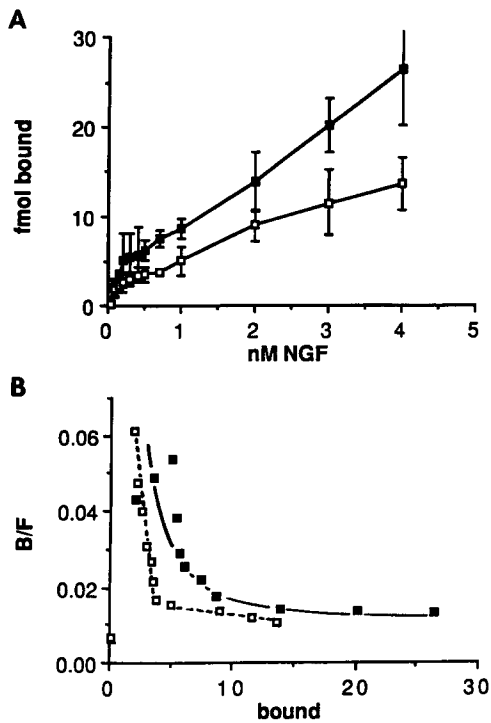
To be sure that the radioactivity migrating with the dye front was iodotyrosine rather than unbound iodine, these peak sections of the gel were extracted with water and the material was analyzed by TLC as described by Wiley and Cunningham (1982). Iodotyrosine was used as a standard and visualized with ninhydrin. Greater than 90% of the radioactivity in the dye front derived from PC12 cells at 30 and 60 min after loading migrates with the monoiodotyrosine peak on TLC. At these times the radioactivity at the dye front is 12.2% and 12.1% of the radioactivity loaded onto the gel.

For the analysis of intermediate degradation products the cells were loaded and chased with NGF as above but after incubation aliquots of cells and medium were added to 0.5 vol of SDS sample buffer (10% SDS, 5%  $\beta$ -mercaptoethanol, 10 mM Tris, pH 6.8), heated to 95°C for 5 min, and loaded onto 15% acrylamide gels (Laemmli, 1970). After electrophoresis the gels were frozen at  $-70^\circ\text{C}$  and exposed to X-ray film.

## Results

### High Affinity NGF Binding to PC12nnr5 Cells

The binding of  $^{125}\text{I}$ -NGF to normal PC12 cells is characterized by kinetic constants that indicate two classes of NGF binding sites of relatively high (type I,  $\sim 0.33$  nM) and low (type II,  $\sim 5$  nM) affinity (Bernd and Greene, 1984). PC12nnr5 cells, on the other hand, do not show high affinity binding of NGF under equilibrium conditions (Green et al., 1986). However, after short exposure to NGF (nonequilibrium conditions), initial measurements showed that significant binding by PC12nnr5 cells was occurring at con-

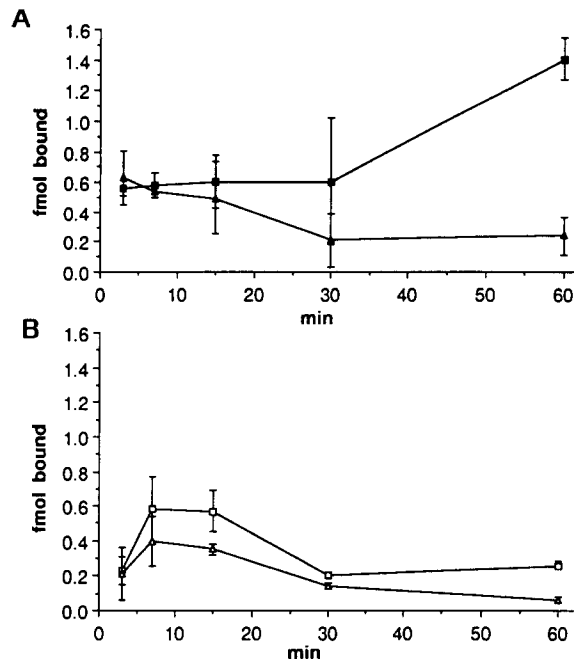


**Figure 1.** Analysis of NGF binding to PC12 and PC12nnr5 cells. (A) Concentration dependence of  $^{125}\text{I}$ -NGF binding to PC12 (■) and PC12nnr5 (□) cells. Cells were incubated with the indicated concentrations of  $^{125}\text{I}$ -NGF in complete medium at  $37^\circ\text{C}$  for 15 min and assayed for bound radioactivity as described in Materials and Methods. Data is expressed as the mean  $\pm$  SD of quadruplicate determinations. (B) Scatchard analysis of the binding of  $^{125}\text{I}$ -NGF to PC12 and PC12nnr5 cells derived from the data in A.

centrations of NGF near the  $K_d$  reported for the high affinity binding site, suggesting that PC12nnr5 cells might possess high affinity (type I) NGF receptors. Fig. 1 A shows that these cells bind NGF at concentrations compatible with the presence of high affinity NGF binding sites similar to those on PC12 cells and that Scatchard analysis of the data (Fig. 1 B) reveals a curvilinear plot for both PC12 and PC12nnr5 cells. These data suggest two binding sites with  $K_d$  values of 0.2 and 4.6 nM for PC12 cells and 0.05 and 1.6 nM for PC12nnr5 cells.

#### Internalization of NGF by PC12nnr5 Cells

In view of previous observations (Eveleth and Bradshaw, 1988) demonstrating that high affinity binding of NGF can result from internalization, the ability of PC12nnr5 cells to endocytose NGF was reexamined. The amount of labeled NGF bound to the surface or internalized (acid-stable) in PC12 and PC12nnr5 cells was measured as a function of time. Fig. 2 shows a plot of NGF bound to the surface (removable by acid washing) and internalized (not removable by acid washing) in PC12 (Fig. 2 A) and PC12nnr5 (Fig. 2 B) cells over the course of a 60-min incubation. As expected, at  $4^\circ\text{C}$ , no significant amount of acid-stable  $^{125}\text{I}$ -NGF is retained by the cells (Table I). When exposed to exogenous NGF, PC12 cells continuously add to their internal pool of NGF for at least 60 min while the amount of NGF bound externally decreases over the course of the experiment, suggesting that NGF-receptor complexes are moving from the



**Figure 2.** Binding and sequestration of NGF. Analysis of  $^{125}\text{I}$ -NGF found on the surface and inside the cell at various times after addition of NGF to PC12 (A) or PC12nnr5 (B) cells. Cells in collagen-coated 35-mm dishes were exposed to 0.2 nM  $^{125}\text{I}$ -NGF in complete medium for the indicated times and processed to measure internal and external NGF by the method of Haigler et al. (1980). Data are expressed as mean  $\pm$  SEM of triplicate determinations. (A) External: ▲; internal: ■. (B) External: △; internal: □.

external (acid-releasable) to the internal (acid-stable) compartment.

PC12nnr5 cells also initially internalize NGF, as judged by the acid stability criterion, but this intracellular accumulation ceases by 15 min and the cells begin to lose intracellular (acid-stable) NGF immediately thereafter. At 60 min, there is no evidence of intracellular accumulation of the ligand. In contrast, the amount of (total) NGF bound to the parental PC12 cells is still rising at 60 min.

#### Degradation of NGF by PC12nnr5 Cells

To ascertain whether the sequestered NGF in PC12nnr5 cells enters the normal intracellular degradation pathway, the conversion of  $^{125}\text{I}$ -NGF to iodotyrosine, a measure of lysosomal degradation, was examined in these cells. In parallel experiments, PC12 cells were loaded with  $^{125}\text{I}$ -NGF for 30 min and PC12nnr5 cells were loaded for 10 or 30 min, and each was chased with cold NGF at the same concentration. At various times after the chase was begun, the cells were detergent solubilized, and the amount of labeled iodine migrating with the dye front in nondenaturing acrylamide gels was used as a measure of the conversion of internalized  $^{125}\text{I}$ -NGF to  $^{125}\text{I}$ -tyrosine. Fig. 3 shows the distribution of radioactivity as a function of migration distance in these gels. As shown in Fig. 4, the summation of the radioactivity migrating at the dye front, which corresponds to  $^{125}\text{I}$ -tyrosine (marked by solid arrows in Fig. 3), shows that PC12 but not PC12nnr5 cells degrade the internalized hormone to amino acids. In the gel system used, intact NGF will not enter the gel and the possibility for incomplete degradation cannot be gauged. A

**Table I. Binding and Sequestration of <sup>125</sup>I-Labeled NGF to PC12 and PC12nnr5 Cells**

Time min	PC12			PC12nnr5		
	Internal	External	I/E	Internal	External	I/E
3	0.56 ± 0.05	0.63 ± 0.18	0.90	0.23 ± 0.08	0.21 ± 0.15	1.07
7	0.58 ± 0.08	0.54 ± 0.02	1.08	0.58 ± 0.19	0.40 ± 0.14	1.44
15	0.60 ± 0.17	0.49 ± 0.24	1.23	0.57 ± 0.12	0.35 ± 0.03	1.62
30	0.60 ± 0.42	0.21 ± 0.18	2.87	0.20 ± 0.01	0.14 ± 0.02	1.41
60	1.41 ± 0.14	0.24 ± 0.13	5.83	0.26 ± 0.02	0.06 ± 0.02	4.73

Internal and external are defined by the acid washing technique of Haigler et al. (1980). Values for Internal and External are in fmol/10<sup>5</sup> cells.

**Table II. Binding and Sequestration of <sup>125</sup>I-Labeled EGF to PC12 and PC12nnr5 Cells**

Time min	PC12			PC12nnr5		
	Internal	External	I/E	Internal	External	I/E
3	3.86 ± 0.44	4.82 ± 0.63	0.80	0.54 ± 0.44	1.07 ± 0.45	0.50
7	9.70 ± 0.88	2.05 ± 0.36	4.73	1.08 ± 0.27	2.33 ± 0.39	0.46
15	13.02 ± 0.84	1.12 ± 0.13	11.63	1.19 ± 0.15	0.94 ± 0.33	1.27
30	15.84 ± 0.86	1.02 ± 0.09	15.53	1.72 ± 0.36	0.63 ± 0.26	2.73
60	18.50 ± 5.33	1.08 ± 0.15	17.13	1.93 ± 0.08	2.54 ± 0.36	0.76

small peak does appear over time in the PC12 but not the PC12nnr5 cells (intermediate between the top and bottom positions) that might represent such an intermediate; however, this was not observed on SDS gel analysis (see below).

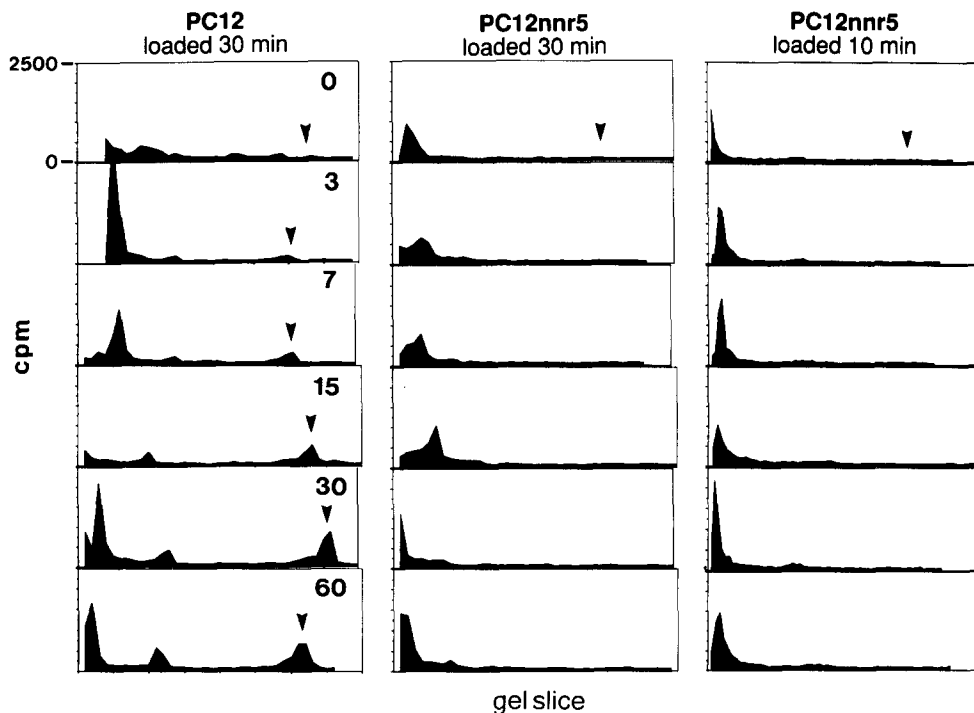
To be certain that the counts migrating with the dye front were actually <sup>125</sup>I-tyrosine, the gel slices were extracted and TLC was performed to separate the iodotyrosine from the iodine (Wiley and Cunningham, 1982). Most (>70%) of the counts migrating at the dye front comigrate with monoiodotyrosine (data not shown), demonstrating that this accumulation of label is due to the degradation of labeled proteins and does not arise from nonspecific trapping of iodine by the cells.

The possibility that the NGF-receptor complexes were

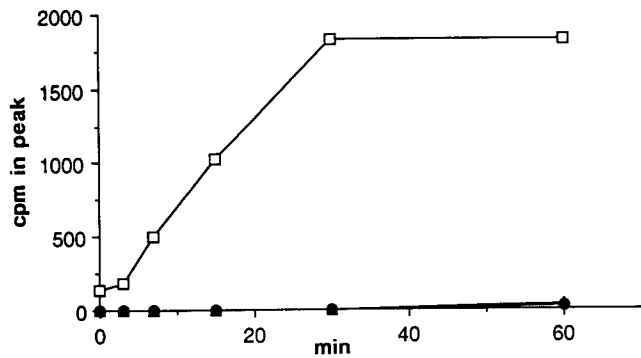
partially degraded and then returned to the surface in these cells was also examined. Fig. 5 shows that no intermediate NGF degradation products can be detected by SDS-PAGE in PC12nnr5 (or PC12) cells up to 60 min after loading with NGF. (No degradation products were detected even after long exposures of this gel). These data further argue that the NGF is not degraded in PC12nnr5 cells.

#### Receptor Depletion

The NGF internalized by the PC12nnr5 cells is released intact into the medium within a short time (~30 min) after internalization. To determine whether NGF binding sites also were returning to the surface, a receptor depletion experi-



**Figure 3.** Degradation of internalized NGF. Cells in suspension were loaded with 0.2 nM <sup>125</sup>I-NGF in complete medium for the indicated times. At the end of this period, cells were washed to remove unbound <sup>125</sup>I-NGF, and incubated in the presence of 0.2 nM unlabeled NGF for the times indicated in each box. Cells and media were made 10 mM in CHAPS and electrophoresed in native acrylamide gels. The gels were sectioned, and the radioactivity in each was determined. Each box represents the radioactivity profile of a single gel. Arrowheads indicate the position of the dye front.



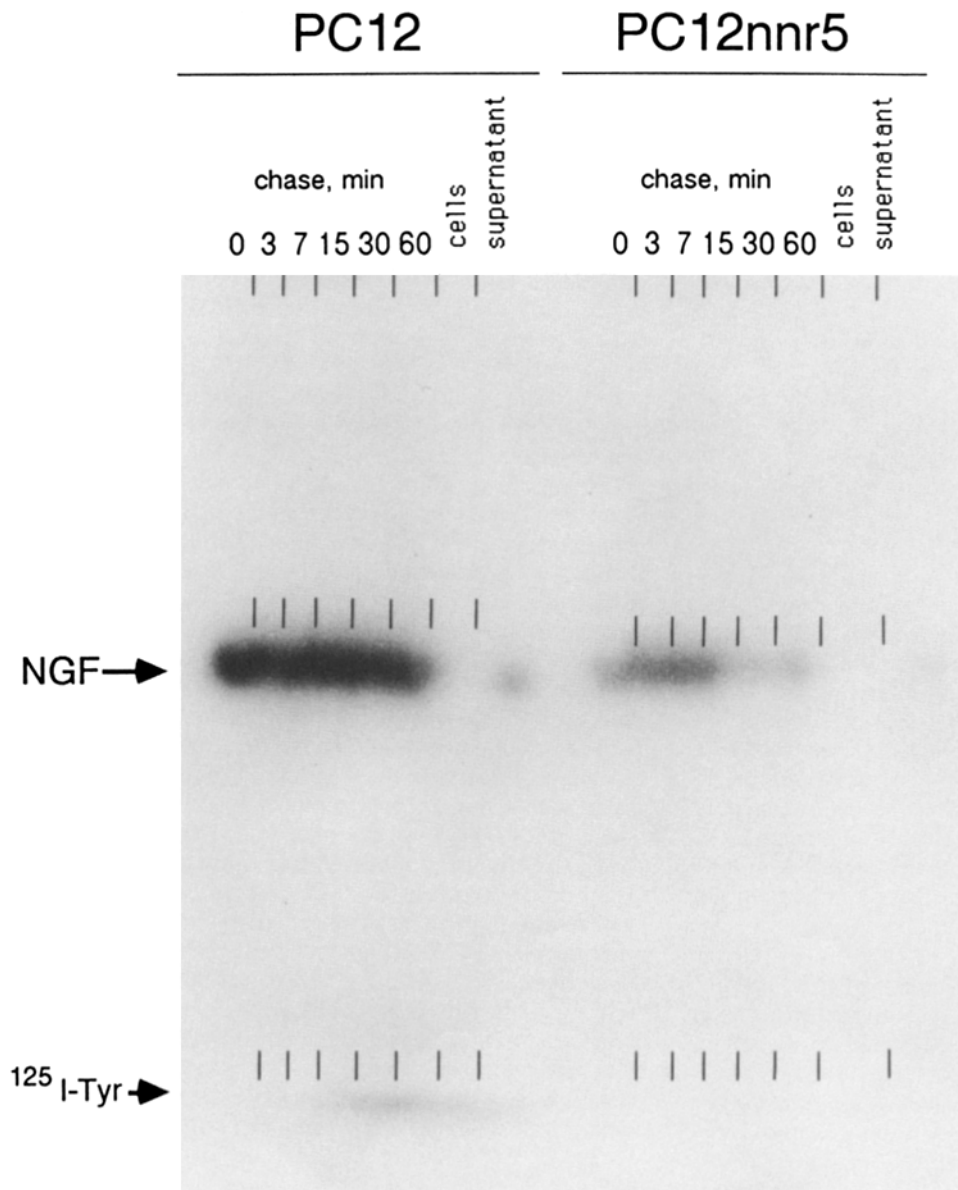
**Figure 4.** Profile of <sup>125</sup>I-tyrosine formation in PC12 and PC12nnr5 cells. Total radioactivity migrating at the dye front in the experiments shown in Fig. 3 are shown. (□) PC12 cells; (●, ▲) PC12nnr5 cells loaded for 30 and 10 min, respectively.

ment over the same time course was performed. The data (not shown) were consistent with the view that some depletion occurred, particularly in PC12nnr5 cells. However, the degree of depletion was somewhat variable preventing any

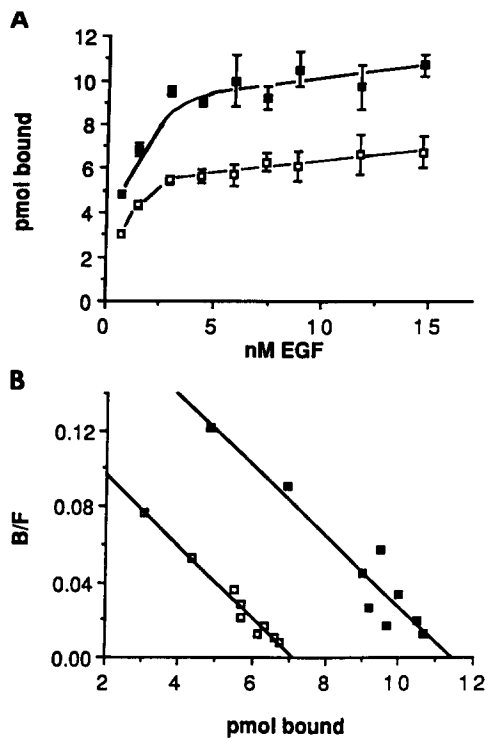
quantitative evaluation. If indeed there is significant loss of surface receptors, it suggests that some proportion is being degraded rather than being recycled after internalization. As another measure of receptor integrity, PC12 and PC12nnr5 cell NGF receptors (p75) were measured by immunoblotting analysis but depletion or degradation in either cell type was not seen.

#### **Binding and Internalization of EGF by PC12 and PC12nnr5 Cells**

To evaluate the possibility that PC12nnr5 cells are generally defective in the internalization of surface ligands by receptor-mediated endocytosis, the interaction of EGF with these cells was examined (see Table II). Fig. 6 A shows the binding of EGF to PC12 and PC12nnr5 cells and a Scatchard analysis of this data (Fig. 6 B). Both cell types possess EGF receptors of similar affinities. The internalization of EGF was assayed in the same way as previously performed for NGF (Fig. 7). While the PC12nnr5 cells internalize EGF more slowly than the parental PC12 cell line, the amount of EGF in the internal compartment of both cell types increases steadily over the



**Figure 5.** Electrophoretic analysis of <sup>125</sup>I-NGF degradation products in PC12 and PC12nnr5 cells. Cells were incubated in suspension in complete medium with 0.2 nM <sup>125</sup>I-NGF for 10 min, washed to remove unbound NGF, and incubated for the indicated periods of time in complete medium containing 0.2 nM unlabeled NGF. At the end of the incubation, the cells and medium were added to SDS sample buffer, boiled for 5 min, and electrophoresed in 15% acrylamide gels according to Laemmli (1970). The gels were frozen at -70°C and exposed to X-ray film. Lanes marked *cells* and *supernatant* represent samples taken at 60 min, after separation by centrifugation.



**Figure 6.** Binding of EGF to PC12 and PC12nr5 cells. The binding of EGF to PC12 (■) and PC12nr5 (□) cells was assayed as described for NGF (see Fig. 1). (A) Concentration dependence of binding; (B) Scatchard analysis of the data in A.

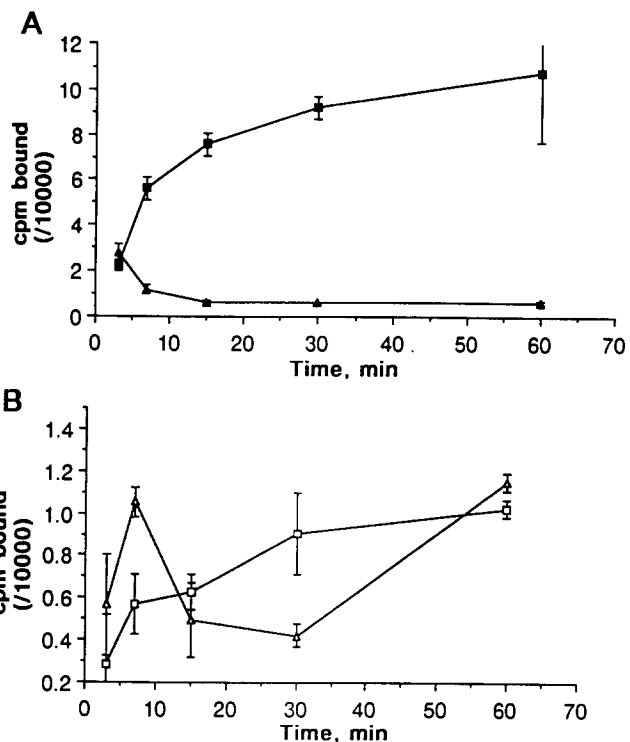
course of the experiment. Interestingly, the PC12nr5 cells display a reproducible increase in cell surface binding at 60 min (Fig. 7 B).

As a further test of this process, the degradation of bound EGF by PC12 and PC12nr5 cells was measured (Fig. 8), as described for NGF. Both cell types degrade the bound EGF to iodotyrosine; however, the PC12nr5 cells are much less efficient at both internalization and degradation of EGF than the parental cell line.

## Discussion

Chemically induced mutant cell lines often provide useful insight into biological mechanisms. In this study, PC12nr5 cells, a variant of the PC12 cell line that is not responsive to NGF, have been analyzed for their ability to bind, internalize, and degrade two ligands, NGF and EGF, that interact specifically with cell surface receptors. In native PC12 cells, these two growth factors have generally opposite effects, inducing neuronal differentiation and mitosis, respectively. However, both factors are readily internalized, as ligand-receptor complexes, and both are rapidly degraded by lysosomal proteases. The role of these processes in the mechanism of either factor is uncertain.

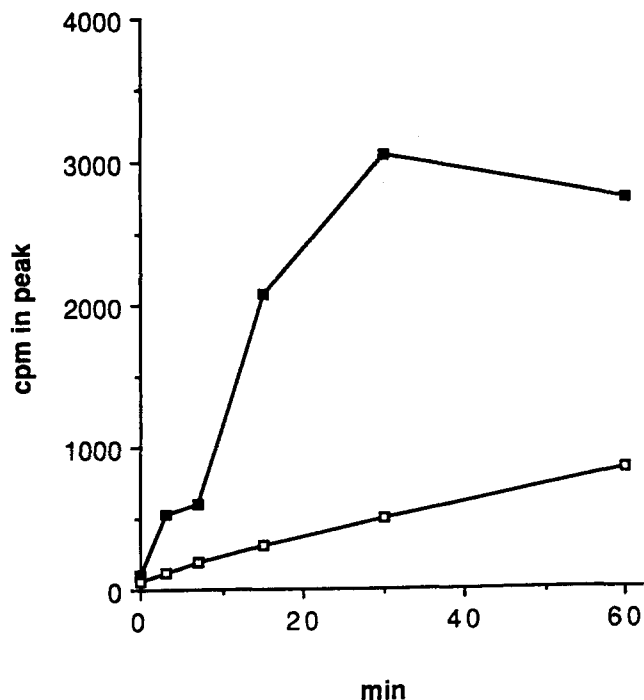
In their initial characterization of PC12nr5 cells, Green et al. (1986) found only low affinity binding of NGF, and did not observe internalization (or degradation) after 60 min. These observations, based on equilibrium measurements, were consistent with the view that only high affinity (type I) receptors can internalize bound NGF and suggest that the defect in these cells was associated with this receptor type.



**Figure 7.** Internalization of EGF by PC12 and PC12nr5 cells. The amount of acid-releasable and acid-unextractable radioactivity associated with PC12 (A) and PC12nr5 (B) cells in the presence of 5 nM  $^{125}\text{I}$ -EGF was assayed as for NGF (see Fig. 2).

However, two observations are not explained by that interpretation. First, these cells are poorly or not at all responsive to basic fibroblast growth factor (bFGF) while native PC12 cells are, differentiating in a manner indistinguishable from that shown for NGF (Togari et al., 1983; Rydel and Greene, 1987). Since the receptors for NGF and bFGF are clearly unique, either the defect that prevents the NGF response must be general enough to also affect the response to bFGF or there are multiple defects that independently affect each factor. Secondly, by examining the effects of several reagents that influence endocytosis on type I and type II NGF binding, we demonstrated that type I receptors could be formed through the reversible endocytosis of occupied type II receptors (Eveleth and Bradshaw, 1988). This concept is supported by other independent observations (Sonnenfeld and Ishii, 1985; Green and Greene, 1986; Kasaian and Neet, 1988; Buxser et al., 1990). Although the impaired response to bFGF could have an alternative explanation, a defect in PC12nr5 cells in only the high affinity receptor is not consistent with a single receptor model.

This study amplifies the observations of Green et al. (1986) and provides a modified, but consistent, explanation for the defect that affects the NGF response. It also adds further insight into the mechanism of NGF-induced signal transduction in PC12 cells and probably responsive neurons. In the main, these results establish that the interaction of NGF with the PC12nr5 cell surface receptor (p75) is unaffected and that high affinity binding can be demonstrated after a short incubation period. This suggests that these cells might also be competent to internalize the receptor-ligand complex, albeit that after a 60-min exposure to  $^{125}\text{I}$ -NGF, as



**Figure 8.** Degradation of internalized EGF. The degradation of EGF by PC12 (■) and PC12nnr5 (□) cells was assayed after loading for 30 min in the presence of 5 nM  $^{125}\text{I}$ -EGF as described for NGF (see Fig. 3). The data shown represents the radioactivity found at the dye front (equivalent to  $^{125}\text{I}$ -tyrosine) as presented for  $^{125}\text{I}$ -NGF degradation in Fig. 4.

reported by Green et al. (1986), no internalization of the hormone is evident. This apparent paradox has been resolved by a more extensive kinetic analysis which showed internalization (or at least sequestration) does occur, in an apparently reversible manner, at an earlier time. Although this does not lead to degradation of NGF, it appears to be sufficient to give rise to measurable high affinity binding. Importantly, these experiments cannot discriminate between a reduced rate of internalization and an increased rate of recycling; either could be responsible for the observed decrease in sequestered NGF.

A prediction of the model that relates high affinity binding to internalization (Eveleth and Bradshaw, 1988) is that, given the behavior of the PC12nnr5 cells, apparent high affinity binding would be maximal at the point where internalization is maximal and would not be observed at times or under conditions when NGF is not sequestered inside the cells. This is consistent with the observations of Green et al. (1986), in which high affinity binding was not observed after incubations were allowed to reach equilibrium.

The identification of high affinity on PC12nnr5 cells has also been observed by Kasaian and Neet (1990). In addition, they concluded that small amounts of NGF were internalized but that it could not be transported or processed normally. However, they did report, in contrast to this study, some lysosomal degradation as judged by TCA precipitation. The small amounts observed were consistent with the substantially decreased cell surface binding of NGF by the mutant cells, leading these workers to suggest that the loss of NGF responsiveness of nnr5 cells may be due to insufficient density of cell surface receptors. The results reported in this study

suggest that the defect affecting the NGF response actually prevents degradation by blocking access to the lysosomal compartment and therefore is more likely to arise from a mutation(s) in a protein essential for receptor-mediated endocytosis of the NGF-receptor complex.

The relationship between NGF receptor structures and their measured affinities remains a source of confusion and controversy, particularly with respect to high affinity interactions. Many cells (both responsive and nonresponsive) express a low molecular weight protein, that has an observed molecular mass of  $\sim 75$  kD (although its actual molecular weight is clearly substantially lower) (Johnson et al., 1986; Radeke et al., 1987). This protein binds NGF with an affinity of  $10^{-9}$  M (as judged by equilibrium and kinetic measurements) (Sutter et al., 1979) and is incapable, by itself, of eliciting responses that depend on signal transduction mechanisms that lead to modulations in gene expression. This protein is present in both PC12 and PC12nnr5 cells. The entity that is responsible for NGF-induced response is of larger size, as judged by covalent cross-linking experiments (Masague et al., 1981; Hosang and Shooter, 1985; Kouchalagos and Bradshaw, 1986). Although there is some variation between target cells, the range of masses identified is  $\sim 130$ – $150$  kD. Direct measurements have indicated this species to be the high affinity receptor (at least in PC12 cells) (Hosang and Shooter, 1985). However, its chemical composition was harder to determine; the majority of evidence favored a complex between p75 and another, unidentified, protein (with an expected molecular mass of  $\sim 60$  kD) (Bothwell, 1991). Whether this complex occurred transiently and whether it possessed intrinsic high affinity binding (or was rather able to achieve it through receptor-mediated endocytosis) remains unresolved.

The identification of the protooncogene *trk* (or *trk A*) as a second NGF-binding entity in some responsive cells (including PC12 cells) has added another dimension to the NGF receptor picture (Kaplan et al., 1991; Klein et al., 1991). This protein is the correct size for the observed high molecular weight species and, indeed, has been so identified in PC12 cells. Further, it has been reported to bind NGF directly with high affinity (Klein et al., 1991). However, other observations suggest that high affinity binding (and biological activity) can only be induced through a complex of p75 and *trk A* (with NGF bound to one or both proteins) (Berg et al., 1991; Hempstead et al., 1991; Yan et al., 1991). As such, it is really only a variation of the "accessory protein" model described above. Although *trk A*, which contains an intrinsic tyrosine kinase, can generate NGF-induced tyrosine phosphorylations itself (when transfected into oocytes) (Nebreda et al., 1991), it has not been shown that these can occur in PC12 cells (in the absence of p75) or that they lead to neuronal differentiation.

The importance of *trk A* to NGF responses is clearly illustrated by the recent demonstrations that PC12nnr5 cells lack this protein and that transfection with a full-length cDNA restores NGF responsiveness (Loeb et al., 1991). This finding also suggests that *trk A* is required for competent internalization to occur (either alone or in concert with p75). However, it is unlikely to explain the lack of response of these cells to bFGF. Although they are unable to differentiate, PC12nnr5 cells do show a normal (as compared to PC12 cells) internalization and degradation of bFGF (Raffioni, S., and R. A.

Bradshaw, unpublished observations) and exhibit similar mechanistic responses (phospholipid hydrolysis (Altin, J. G., and R. A. Bradshaw, unpublished observations) and immediate-early response gene induction) (Altin et al., 1991). Since the FGF receptor family is more complex, there may be a defect in one or more of these species that would affect the neuronal-like responses but still allow internalization and other general responses to occur.

The fate of the receptor components during the endocytosis of NGF-receptor complexes may be variable. Buxser et al. (1990) have argued that the recycling of NGF receptors occurs in PC12 cells, which is consistent with our observations (Eveleth and Bradshaw, 1988). In these studies, some evidence for receptor depletion (as measured by NGF binding) was observed, particularly with PC12<sup>nnr5</sup> cells which, because of their altered state, may treat the internalized (or sequestered) receptors differently than native PC12 cells. In the absence of more quantitative data, this issue can not be specifically resolved. It is, however, entirely possible that the *nnr5* NGF receptors, because of their defective nature, are specifically removed in some fashion while the intact ligand is returned to the cell surface.

The requirement for internalization of NGF as part of its mechanism is also consistent with the long-held view (for review see Greene and Shooter, 1980) that NGF, at least in the peripheral nervous system, acts via retrograde axonal transport after specific internalization at presynaptic membranes. The transported vesicles may not generate a signal until they reach the perikaryon and can associate with one or more signaling molecules located there but not in the synaptic ending. By analogy with PC12 cells, this would not include the accessory protein which would presumably be required at the membrane to permit internalization to occur. Such a model would be entirely consistent with the special requirements of neurons to communicate between synapse and cell body and would perhaps explain why the NGF receptor lacks a tyrosine kinase domain as an inherent part of its structure.

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