Long-Term, Heterologous Down-Regulation of the Epidermal Growth Factor Receptor in PC12 Cells by Nerve Growth Factor

Philip Lazarovici, Geneva Dickens, Hiroshi Kuzuya, and Gordon Guroff

The Section on Growth Factors, National Institute of Child Health and Human Development, National Institutes of Health, Bethesda, Maryland 20892

Abstract. Cells of the rat pheochromocytoma clone PC12 possess receptors for both nerve growth factor (NGF) and epidermal growth factor (EGF), thus enabling the study of the interaction of these receptors in the regulation of proliferation and differentiation. Treatment of the cells with NGF induces a progressive and nearly total decrease in the specific binding of EGF beginning after 12 h and completed within 4 d. Three different measures of receptor show that the decreased binding capacity represents, in fact, a decreased amount of receptor: (a) affinity labeling of PC12 cell membranes by cross-linking of receptor-bound 125I-EGF showed a 60-90% decrease in the labeling of 170- and 150-kD receptor bands in cells treated with NGF for 1-4 d; (b) EGF-dependent phosphorylation of a src-related synthetic peptide or EGF receptor autophosphorylation with membranes from NGF-differentiated cells showed a decrease of 80 and 90% in the tyrosine kinase activity for the exogenous substrate and for receptor autophosphorylation, respectively; (c) analysis of 35S-labeled glycoproteins isolated by wheat germ agglutinin-Sepharose chromatography from detergent extracts of PC12 membranes showed a 70-90% decrease in the 170-kD band in NGF-differentiated cells. These findings permit the hypothesis that long-term heterologous down-regulation of EGF receptors by NGF in PC12 cells is mediated by an alteration in EGF receptor synthesis. It is suggested that this heterologous down-regulation is part of the mechanism by which differentiating cells become insensitive to mitogens.

The rat pheochromocytoma clone PC12 has been used as a model of neuronal differentiation because the cells acquire neuronal properties and stop dividing in response to nerve growth factor (NGF) (Greene and Tischler, 1976). The differentiation induced by NGF appears to be reversible, but, except for this, the changes that occur lead to cells with the phenotype of sympathetic neurons (Kimhi, 1981). The PC12 cells, of course, carry NGF receptors (Herrup and Thoenen, 1979; Landreth and Shooter, 1980) and the binding of NGF to these specific receptors triggers many and diverse arrays of cellular responses, including both short-term, rapid membranal changes, and long-term alterations in cell properties associated with changes in transcription (Guroff, 1983). Among these responses, NGF-induced differentiation in PC12 involves a transition from a mitotic to a nonmitotic state.

In this regard, it was of interest to observe that the PC12 cells also have receptors for epidermal growth factor (EGF) (Huff and Guroff, 1979; Huff et al., 1981; Boonstra et al., 1985). This peptide is a potent mitogen for many of the cells with which it interacts, and is a mild mitogen for PC12 (Huff et al., 1981). Since the cells, then, have receptors for both a differentiating agent and a mitogen, experiments in this laboratory have been designed to explore what happens when the cells are treated with both agents.

In previous studies it has been shown (Huff and Guroff, 1979; Huff et al., 1981) that treatment of the cells with NGF reduces the amount of EGF binding to the cells by 80% or more. Scatchard plots have indicated that the number of EGF receptors decreases, but that the affinity of the remaining receptors for EGF remains constant. The data also reveal that the decrease in receptor number is gradual, progressing over a period of several days. These findings suggest that the changes in the EGF receptor are part of the long-term alterations caused by NGF and perhaps involve transcriptional regulation.

In this study, the long-term alteration in EGF-binding capacity of PC12 is characterized further and shown to be related to an altered concentration of receptor, thus appearing to be a novel form of heterologous EGF receptor regulation. The functional implications of this phenomena with respect to the control of proliferation and differentiation in PC12 cells are discussed.

1. Abbreviations used in this paper: EGF, epidermal growth factor; NGF, nerve growth factor.
Materials and Methods

Materials

NGF was prepared by the method of Bocchini and Angeletti (1969) and EGF by the method of Savage and Cohen (1972). EGF was also obtained from Collaborative Research Inc. (Lexington, MA). For selected experiments the NGF was further purified by reverse-phase HPLC as described by Petrides and Shooter (1986) using a Spherisorb ODS 2 column (LKB Ultrapac, 3 μm; LKB Instruments, Inc., Gaithersburg, MD). Two linear gradients were applied, 10-35 and 35-80% acetonitrile in 1% trifluoroacetic acid, for 45 min. Peaks were monitored at 226 nm. The peak fractions were collected and concentrated, and the NGF content evaluated by the ability of the materials to induce neurite formation by PC12 cells and by their ability to compete for the NGF receptor on PC12 cells. Using identical chromatographic conditions, the EGF was resolved into α- and β-EGF as described by Matrisian et al. (1982). Cytochrome C, phenylmethylsulfonyl fluoride (PMSF), aproton, A23187, choline chloride, and phosphol 12-merystate 13- acetate were purchased from Sigma Chemical Co. (St. Louis, MO). Sodium vanadate was obtained from Fisher Scientific Co. (Pittsburgh, PA). Polyclonal antibodies (Duan et al., 1986) and monoclonal 125-IgG antibodies (Chandler et al., 1985) against the rat EGF receptor were a gift of Drs. W. A. Dunn and A. L. Hubbard (Department of Cell Biology and Anatomy, The Johns Hopkins University School of Medicine, Baltimore, MD). Antiser raised against NGF and EGF were purchased from Collaborative Research Inc. Electrophoresis reagents and molecular weight standards were obtained from Bio-Rad Laboratories (Richmond, CA). All other reagents were of the highest quality available.

Cell Culture

PC12 cells were cultured at 37°C as monolayers in 150-cm² culture flasks in DMEM (Gibco, Grand Island, NY) supplemented with 7% FBS, 7% horse serum, and 100 μg of streptomycin/ml. The cells were split in a 1:4 or 1:6 ratio each week and the medium changed once during the week. Human carcinoma A431 cells and rat kidney VB4T cells (obtained from Dr. J. DeLarco, Laboratory of Viral Carcinogenesis, National Institutes of Health) were grown in the same medium. Hybridoma cells yielding 125-IgG were grown as previously described (Chandler et al., 1985).

Plasma Membrane Preparation and Solubilization

Cells were harvested in 10 ml of borate buffer (0.05 M borate, 0.15 M NaCl, 1 mM MgCl₂, pH 7.3), and collected by low-speed centrifugation. The cell pellet was washed twice with PBS (Ca²⁺ and Mg²⁺ free) containing 2 mM PMSF and 0.1% aprotinin. The cells were disrupted at 4°C by high speed polytron homogenization for 10 s followed by 10 strokes of mechanical homogenization. The extent of lysis was monitored by microscopic examination. One-half volume of 60% sucrose in PBS was added and the homogenate was centrifuged at 500 g for 10 min to remove intact cells and nuclei. The clarified lysate was layered on 35% sucrose in PBS and centrifuged at 25,000 g for 1 h. The membranal band on the surface of the sucrose was harvested, resuspended in a convenient volume of PBS, and the membranes were collected by centrifugation at 100,000 g for 10 min at 4°C. Protein concentration was determined by the Lowry method (Lowry et al., 1951), the membrane protein was adjusted to a concentration of 1 mg/ml, and the suspension stored at −70°C. Membrane solubilization was performed with 1% Triton X-100 plus 1% SDS in 20 mM N-2-hydroxyethyl-N'-2-piperazine-ethane sulfonic acid (Hepes), pH 7.4, containing 10% glycerol, 0.15 M NaCl, 0.2 mM PMSF, 1% aprotinin, and 1 mM EDTA. Insoluble material was removed by centrifugation for 20 min at 100,000 g.

Indication of Growth Factors and Binding Assays

Growth factors were iodinated by the solid phase lactoperoxidase method and the specific binding was measured by binding assay. The results were expressed as the mean ± the SD. Binding studies done using plasma membrane preparations were performed similarly, at 4°C, except that free- and membrane-bound factors were separated by microfuge centrifugation for 10 min at 4°C (Beckman Instruments, Inc., Palo Alto, CA). Binding buffers used for membrane assays, unless otherwise stated, consisted of either PBS or 50 mM Tris-HCL containing 2 mg/ml of ovalbumin, at pH 7.4. 125-I-EGF binding to the surface of PC12 cells after papain proteolysit or as a function of pH was done as described by DiPaola and Maxfield (1984). PC12 monolayers were rinsed with PBS, and incubated for 10 min at room temperature in PBS containing 1 μg/ml of activated papain. The cells were then rinsed free of papain by washing three times with PBS and tested for 125-I-EGF binding.

Homo-Down-Regulation of EGF Receptors and Determination of EGF Degradation

Plasma membrane EGF receptors were down-regulated by incubation of the cell monolayers with unlabeled EGF (10 ng/ml) for different periods of time. Monolayers were washed twice with PBS (Ca²⁺ and Mg²⁺ free) and incubated in fresh binding buffer (2 h at 37°C) to allow internalization of the remaining EGF-receptor complexes. After two additional washes with binding buffer the extent of receptor down-regulation was determined by measuring the amount of 125-I-EGF binding.

Measurement of the cell-mediated degradation of 125-I-EGF by PC12 cells was done as described by Layer and Shooter (1982); 10% TCA was used to precipitate the radioactive material in the incubation medium.

Receptor Affinity Labeling

PC12 membranes (300-500 μg protein) or A431 carcinoma or kidney cell membranes (50 μg) were incubated in PBS containing 0.1% ovalbumin at 4°C for 60 min with 5 nM 125-I-EGF in the absence of any other ligand in the presence of 5 μM unlabeled EGF or 1 μM 125-IgG. The membranes were washed intensively with binding buffer, resuspended in 1 ml of binding buffer containing 20 μl of 25 mM disuccinimidyl suberate (Pierce Chemical Co., Rockford, IL), dissolved in dimethyl sulfoxide, and incubated for 10 min at 5°C. The membranes were washed twice with binding buffer and twice with 0.25 M sucrose containing 10 mM Tris and 1 mM EDTA, pH 7.0, and solubilized by heating for 10 min at 100°C with 100 μl of double strength electrophoresis sample buffer. Insoluble material was removed by centrifugation at 12,000 g for 10 min and the samples were analyzed by electrophoresis on SDS polyacrylamide gels. The efficiency of affinity labeling of the membranes using this procedure is ~10%.

Tyrosine Kinase Assays

Measurement of EGF-dependent tyrosine kinase activity was performed by the method of Cohen (1983). PC12 cell membranes (200 μg of protein) or A431 or kidney cell membranes (20 μg) were preincubated with EGF (50 ng/ml) for 15 min at 40°C in a reaction buffer containing 20 mM Hepes-NaOH, pH 7.2, 0.5 mM MgCl₂, 3 mM MnCl₂, and 50 μM Na₂VO₄ in a final volume of 50 μl. The kinase reactions were initiated by the addition of 2 μCi of γ-³-P-ATP in 50 μl unlabeled ATP, and continued for 2 min, unless otherwise stated, at 4°C. The reactions were stopped by the addition of SDS sample buffer and the tubes boiled for 10 min. The samples were analyzed by SDS PAGE and autoradiography. Detection of the phosphotyrosine content of the EGF receptor was done by incubation of the polyclonal antibodies in alkalai as detailed by Cooper et al. (1983) to hydrolyze phosphoserine. The radioactivity contained in the EGF receptor band was estimated by densitometric scanning of the resultant autoradiograms and by exclusion of the approximate portion of the dried gel and subsequent analysis by liquid scintillation spectrometry. When the src-related synthetic peptide Ac-Arg-Asp-Glu-Asp-Ala-Glu-Tyr-Ala-Ala-Arg-Gly (100 μM) (Peninsula Laboratories, Inc., Belmont, CA) was used as a substrate, the reaction was terminated by application of the sample to PBI phosphocellulose paper (Whatman Inc., Clifton, NJ). The paper was washed with 5% phosphoric acid, then with 20% ethanol. The papers were dried and the radioactivity counted in a liquid scintillation counter.
Metabolic Labeling and Membrane Solubilization

Slightly subconfluent PC12 and A431 cells were labeled by incubating the cells for 18 h in methionine-free DME medium containing 60 μCi/ml L-[35S]methionine (Amersham Corp., Arlington Heights, IL). To prepare labeled membranes, the cells were washed three times with cold PBS and homogenized and centrifuged as previously described. 35S-Labeled membranes were solubilized by treatment for 20 min at 4°C in RIPA buffer (10 mM Tris, pH 8.5, containing 0.15 M NaCl, 1 mM EDTA, 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS, 1 mM PMSF, and 1 mg/ml aprotinin). One ml of RIPA buffer was added for each mg of protein. Insoluble material was removed by centrifugation at 100,000 g for 10 min. The solubilized supernatants were either subjected to lectin chromatography (or immunoprecipitation) immediately or stored at -70°C for later analysis. Immunoprecipitation of human EGF receptors from [35S]methionine-labeled A431 cells was done with PK-2 antisera by the methods of Kris et al. (1985). The immunoprecipitate was used as a reference for the identification of the EGF-receptor bands on gels.

Wheat Germ Lectin-Sepharose Chromatography

The chromatography was carried out with slight modifications of a previously described procedure (Cohen et al., 1982). Wheat germ lectin-Sepharose (100 μl of gel packed into 1 ml Eppendorf pipette tips plugged with glass wool) was equilibrated with 10 ml of 20 mM Hepes buffer, pH 7.4, containing 0.2% Triton X-100, 10% glycerol, 0.5 mM EGTA, 0.5 mM EDTA, 0.1 mM PMSF, and 50 μM Na3VO4. 200-μl portions of RIPA extracts of [35S]methionine-labeled or unlabeled membranes (450 μg of protein) were applied to the gels; the gels were then washed by centrifugation five times with 1 ml portions of equilibration buffer. Then the gels were either eluted with N-acetylglucosamine (300 mM) in the equilibration buffer for desorption of the bound glycoproteins or dissolved directly in double strength SDS sample buffer and held at 100°C for 10 min. Samples of chromatographed, unlabeled membranes were analyzed for protein kinase activity.

Figure 1. Time course of the NGF-mediated hetero-down-regulation of 125I-EGF binding to PC12 cells. Cells were grown in control medium (o - - - o) or in medium with 2 × 10^-9 M NGF, in the presence (△ - - - △) or absence (○ - - - ○) of anti-EGF antiserum (135 μg/ml). Binding of 125I-EGF (0.2 × 10^6 cpm/10^5 cells) was measured by incubation for 45 min at 37°C at the indicated time points, in the presence or absence of excess unlabeled EGF (10^-9 M). The cells were exposed to NGF (NGF, arrow) 1 d after splitting and fresh NGF was added every 48 h. 5 d later, in a separate experiment, the cells were washed (W, arrow) with fresh medium and cultured in the absence of NGF before the binding assay. Each binding point represents the mean ± SD of two experiments performed in triplicate. The upper inset shows control PC12 cells (left) and typical process outgrowth induced by 2 × 10^-8 M NGF after 2 (center) and 4 (right) d of treatment. The lower inset shows the blocking effect of anti-EGF antiserum on the down-regulation of EGF receptors by EGF. PC12 cells were incubated at 37°C for the indicated time periods with EGF (0.2 nM) in DME. Monolayers were washed to remove the unbound factor and incubated in fresh DME (1 ml) for 2 h at 37°C to internalize the remaining EGF-receptor complexes. After two additional washes with medium, the extent of receptor down-regulation was determined by measuring the amount of specific 125I-EGF binding under conditions detailed in Materials and Methods.
**SDS PAGE and Autoradiography**

Gel electrophoresis was performed for 4 h at 100 V according to Laemmli (1970) using a 3% stacking gel and a 7.5% separating gel. After electrophoresis, the gel was stained with 0.05% Coomassie Blue in 45% methanol, 9% acetic acid, and destained in 10% acetic acid, 10% n-propanol. Gels of [35S]methionine-labeled membranes were treated with enhancer (Enlightening; New England Nuclear, Boston, MA) for 30 min. The gels were dried under vacuum and exposed to Kodak XAR-5 film at -70°C using an intensifying screen (DuPont Lighting Plus; DuPont Co., Diagnostic and Bio-Research Systems, Wilmington, DE). Autoradiograms were scanned with a laser densitometer (Zeineh Soft Laser Densitometer; LKB Instruments, Inc., Uppsala, Sweden) and the bands corresponding to the EGF receptor were cut out, treated with 500 μl of NCS tissue solubilizer, and then counted in 10 ml of scintillant (Aquasol; Amersham Corp., Arlington Heights, IL).

**Estimation of Membrane Potential and Depolarization**

The estimation of PC12 membrane potential was performed according to an established procedure (Milligan and Strange, 1984). Uptake of 4°C-tetraphenylphosphonium bromide into control and NGF-differentiated PC12 cells suspended in choline buffer (120 mM choline chloride, 5 mM KCl, 2 mM CaCl2, 1 mM MgCl2, 0.2 mM Tris-HCl, 10 mM glucose, pH 7.4) was initiated by the addition of 0.1 μCi of [35S]tetraphenylphosphonium bromide (60 nM) and the incubation continued for 45 min at 37°C. The monolayers were washed with choline buffer containing 0.1% BSA and cell-associated radioactivity was estimated after the addition of scintillant. Depolarization of the cells was done by replacement of choline with the indicated concentration of potassium.

**Results**

**Time Course and Specificity of Reduction in EGF-binding Capacity**

The capacity of NGF-differentiated PC12 cells to interact with 125I-EGF was examined relative to the NGF-induced changes in morphology. The results from such an experiment are shown in Fig. 1. Treatment of the cells with NGF significantly reduced the binding capacity of the cells for EGF after a latency period of 12-24 h. After 5-7 d of NGF treatment a near-total disappearance of EGF-binding capacity was observed. Withdrawal of NGF from the culture medium was accompanied by a gradual reappearance of EGF-binding capacity (Fig. 1). The initial decline in EGF-binding capacity correlates in a general way with the appearance of the NGF-induced morphological changes, e.g., cell hypertrophy, process formation (Fig. 1, top), properties typical of terminally differentiated neurons (Guroff, 1983). The effect of NGF on the EGF-binding capacity of the differentiated cells is concentration dependent and maximal around 2 × 10^-9 M (Fig. 1).

The effect of NGF treatment on EGF binding is unaffected by the presence of an excess of anti-EGF antibodies in the culture medium during the treatment (Fig. 1) or by a preincubation of the NGF preparation with these antibodies (data not shown). The concentration of anti-EGF antibodies used in the former experiment was sufficient to block the homodown-regulation of EGF receptors by EGF (Fig. 1). Furthermore, the reduction in EGF binding caused by treatment with the standard preparation of NGF was also caused (Table I) by treatment with an HPLC-purified peak of NGF eluted with 33-35% acetonitrile (Petrides and Shooter, 1986) with a retention time of 32.2 min. The reduction in EGF-binding capacity produced by treatment with the HPLC-purified NGF could be blocked completely by anti-NGF antiserum, but not by anti-EGF antiserum (Table I). These results, as well as the absence of any competitive inhibition by the NGF preparation (up to 10^-6 M) of EGF binding to PC12 membranes (data not shown), clearly show that the down-regulation of EGF receptors by treatment with NGF is not due to contamination of the NGF preparation with EGF.

This EGF receptor modulation is quite specific for NGF (Table II). Neither cytochrome C nor insulin affected the EGF-binding capacity of PC12 cells. Furthermore, fibroblast growth factor, which also causes the formation of cell processes in PC12 cells (Togari et al., 1985), did not induce a reduction in the EGF-binding capacity. This NGF-induced reduction in EGF binding has quite different characteristics than does the NGF-induced down-regulation of EGF receptors (Fig. 2). At a high NGF concentration, a rapid down-regulation of NGF receptors was seen; this was followed by a gradual recovery process in which ~50% of control binding levels were observed after 3 d. A second addition of NGF caused another rapid down-regulation of the receptors. Therefore, under comparable conditions the reduction in EGF-binding capacity is much slower than the reduction in NGF binding. This is consistent with previous experiments showing that NGF receptors and EGF receptors are separate entities in PC12 cells (Huff et al., 1981) as they are in other cells.

**NGF-induced Reduction in EGF-binding Capacity Is Seen with Isolated Membranes**

A progressive reduction in EGF-binding capacity was seen when binding was measured with plasma membranes iso-

**Table I. Effect of Antibodies on NGF-induced Hetero-Down-Regulation of EGF Receptors in PC12 Cells**

| Antiserum                  | Neurite formation | 125I-EGF specific binding (percent of control) |
|---------------------------|-------------------|-----------------------------------------------|
| µg/ml                     |                   |                                               |
| Anti-NGF antiserum (200)  | ±                 | 92.5                                          |
| Anti-EGF antiserum (135)  | +++               | 36.0                                          |

PC12 cells were treated for 3 d with 2 × 10^-4 M HPLC-purified NGF in the presence or absence of various antisera. Neurite formation and 125I-EGF binding to the washed cells was measured as described in Materials and Methods. The data on EGF binding represents the mean of triplicates and is presented as a percentage of the specific 125I-EGF binding to control cells.

**Table II. Specificity of NGF-induced Hetero-Down-Regulation of EGF Receptors in PC12 Cells**

| Compound tested                       | Concentration | Neurite formation | 125I-EGF specific binding |
|---------------------------------------|---------------|-------------------|---------------------------|
|                                       | M             | fmol/µg protein   |                           |
| None                                  | -             | -                 | 5.8 ± 0.3                 |
| Cytochrome C                          | 10^-6         | -                 | 5.6 ± 0.2                 |
| Insulin                               | 10^-6         | +                 | 6.5 ± 0.5                 |
| Fibroblast growth factor              | 10^-4         | +                 | 6.0 ± 0.3                 |
| NGF                                   | 10^-4         | +++               | 3.8 ± 0.2                 |

Cells were treated for 4 d. Binding of 125I-EGF to the washed cells was measured as described in Materials and Methods. Each number represents the mean ± SD of four determinations.
Figure 2. Time course of NGF-receptor homo-down-regulation in PC12 cells. Cells were grown under conditions identical to those described in Fig. 1. Specific binding of $^{125}$I-NGF (0.3 × 10$^6$ cpm/10$^5$ cells) was measured by incubation for 30 min at 37°C at the indicated time points. The cells were exposed to NGF (NGF, arrow) 1 d after splitting at a high (2 × 10$^{-8}$ M, • - - - •) or a low (2 × 10$^{-10}$ M, △ - - - △) concentration. After 2 d of NGF treatment, half of the cultures received a new dose of 2 × 10$^{-8}$ M NGF (NGF, arrow, dotted line), before the binding assay.

Characterization of the NGF-induced Decrease in EGF-binding Capacity of Differentiated PC12 Cells by Cross-linking

To obtain further proof that a reduction in the number of EGF receptors is the cause of the reduced binding capacity of PC12 cells differentiated with NGF, EGF was cross-linked to its receptors with succinimidyl suberate. Membranes from control and from differentiated cells were isolated, carefully normalized for protein, cross-linked with bound $^{125}$I-EGF, and analyzed by electrophoresis and autoradiography (Fig. 4). Cross-linking of $^{125}$I-EGF to membranes from control PC12 cells resulted in the labeling of a major component of 170,000 D and a minor component of 150,000 D; the pattern was identical to that obtained with membranes from A431 cells (Fig. 4, -EGF, A431 and PC12, lane C). In A431 cells it has been shown that a proteolytic product of the 170,000-D EGF receptor is a species of 150,000 D (Casel and Glaser, 1982). Complete disappearance of the labeling of these bands in the presence of excess unlabeled EGF demonstrated that they indeed represent $^{125}$I-EGF/EGF receptor complexes (Fig. 4, +EGF, A431 and PC12, lane C). Formation of the 170,000- and 150,000-D $^{125}$I-EGF/EGF receptor complexes was also inhibited by ∼80% in the presence of the monoclonal antibody 151-IgG that was recently observed to be a competitive inhibitor of EGF binding to PC12 cells (data not shown) (Chandler et al., 1985); this data supports the specificity of the cross-linking to the EGF receptor. Analysis of the cross-linked EGF receptor bands in PC12 cells treated with NGF for 1 d indicates 50 and 53% reduction in the labeling intensity of the 170,000- and 150,000-D species, respectively (Fig. 4, -EGF, PC12, lane 1). After 2 d of NGF treatment the intensity of labeling of both EGF receptor bands was decreased >90% (Fig. 4, -EGF, PC12, lane 2), and after 4 d of NGF treatment the receptor bands represent only a few percent of the values obtained with control cells (Fig. 4, -EGF, PC12, lane 4). This decrease in labeling is
consistent with a lower number of EGF receptors in NGF-differentiated PC12 cells.

Characterization of EGF-stimulated Tyrosine Kinase Activity in Control PC12 Cell Membranes

Another reflection of an NGF-induced decrease in EGF receptor number would be a decrease in EGF-stimulated tyrosine kinase activity in differentiated PC12 cells. Because tyrosine kinase activity is very low in PC12 cells and the alkaline lysis method commonly used in EGF receptor studies is damaging to PC12 cell receptor activities (unpublished data), we have searched for conditions that are optimal for the measurement of tyrosine kinase activity and, at the same time, under which tyrosyl phosphatases are inhibited. The EGF receptor kinase activity has been shown to be enhanced (relative to serine or threonine kinases) when reactions are performed in the presence of manganese rather than magnesium (Carpenter et al., 1979) and in the presence of vanadate, a protein phosphatase inhibitor (Brautigan et al., 1981; Swarup et al., 1982). As shown in Fig. 5, EGF receptor autophosphorylation in control PC12 cell membranes is stimulated severalfold by EGF when the phosphorylation assay media contained manganese and vanadate (Fig. 5, lane 2); in the absence of manganese (lane 3), vanadate (lane 4), or EGF (lane 8) the autophosphorylation is much lower. The ability of EGF to stimulate EGF receptor autophosphorylation was found to be dose dependent (Fig. 5, lanes 5–7) and almost maximal at $5 \times 10^{-9}$ M EGF (Fig. 5, lane 5). The specificity of the EGF stimulation of EGF receptor autophosphorylation is quite strict; no stimulation was observed upon addition of FGF, NGF, or insulin (data not shown). The rate of autophosphorylation of the EGF receptor increased linearly with increasing concentration of membranes up to 2 mg/ml after which no further increase was observed in the 2-min assay (data not shown). The time course of EGF-stimulated receptor autophosphorylation in vitro in control PC12 cell membranes is linear for ~5 min when measured at a low temperature and a high ATP concentration (data not shown). The basal autophosphorylation activity of the EGF receptor continued to increase during the incubation and after 10 min was very close to EGF-stimulated values. Results from six different batches of control PC12 cell membranes indicate a basal EGF-receptor autophosphorylation activity of $20 \pm 7\%$ of the total receptor tyrosine kinase activity and a five- to 10-fold stimulation by EGF compared with basal activity when measured in the 2 min assay. The effect of EGF on the tyrosine kinase activity of the receptor was seen even more clearly after the gels were exposed to alkali (Fig. 6), which preferentially hydrolyzes phosphoserine. Comparison of identical gels before (Fig. 6 A, lane 2) and after (Fig. 6 B, and 2) alkaline treatment found that 75% of the $^{32}\text{P}$ content of the receptor band remained. These experiments clearly indicate that most of the phosphorylation in the 170,000-D EGF receptor band is on tyrosine residues. In the phosphorylation experiments there is always EGF-dependent phosphorylation of some additional membranal tyrosine-containing proteins of ~30,000–45,000 D; these resemble the well-documented EGF-receptor “substrates” in A431 cells (Fava and Cohen, 1984). It is important to emphasize the absence of any detectable effect of NGF on the phosphorylation of membranal proteins or the synthetic peptide (data not shown), thus distinguishing the NGF receptor from the EGF receptor and from the other polypeptide growth factor receptor kinases. Since EGF-stimulated receptor autophosphorylation might have different properties than the phosphorylation of physiological or exogeneous substrates (Hunter and Cooper, 1985), the phosphorylation of a src-related synthetic peptide by PC12 cell membranes was also studied; such peptides are commonly used as substrates for EGF-stimulated tyrosine protein kinase phosphorylation by cell membranes (Pike et al., 1982). EGF stimulates the phos-

Figure 6. Tyrosine-specific autophosphorylation of EGF receptor from PC12 cells. EGF-stimulated phosphorylation of (J) kidney cell, (2) control PC12 cells, and (3 and 4) NGF-treated cell membranes analyzed (A) before and (B and C) after alkaline treatment of the polyacrylamide gels. Phosphorylation conditions and alkaline treatment were as described in Materials and Methods. Membrane protein: lane 1, 60 µg; lane 2, 225 µg; lane 3, 225 µg; lane 4, 450 µg; Autoradiogram exposure: A, 4 h; B, 12 h; C (the same gel as B), 48 h.
phorylation of the src-peptide by membranes from control
by PC12 and Kidney Cell Membranes

Table III. Comparison of EGF-activated Phosphorylation
by PC12 and Kidney Cell Membranes

| Preparation       | Tyrosine kinase specific activity |
|-------------------|----------------------------------|
|                   | Autophosphorylation | Peptide phosphorylation |
|                   | (cm²/min/mg protein) | (pmol/min/mg protein) |
| PC12, control cells | 0.32 ± 0.07           | 9.0 ± 1.5              |
| PC12, NGF-treated cells | 0.04 ± 0.01          | 2.0 ± 0.7              |
| VB4T kidney cells  | 12.00 ± 0.14          | 289 ± 14               |

PC12 cell membranes (225 µg protein) or VB4T cell membranes (30 µg protein) were incubated for 2 min at 24°C in a final volume of 50 µl with 50 µm ATP containing 2 µCi γ-32P-ATP, and the assay buffer as detailed in Materials and Methods. For autophosphorylation measurements the reactions were terminated by the addition of double strength SDS-sample buffer and subjected to SDS electrophoresis and autoradiography. The EGF receptor (170,000-D band) autophosphorylation was calculated from the peak area of the densitometer tracing of the autoradiograms. Phosphorylation of the tyrosine-containing peptide (100 µm) was allowed to proceed for 10 min at 0°C and terminated by applying 40-µl portions to filter paper (Whatman, Inc.) as described in Materials and Methods. Phosphorylation experiments were assayed in the presence (total activity) and absence (basal activity) of EGF (50 ng/ml) that was preincubated with the membranes for 5 min at 4°C. The basal activities for peptide phosphorylation were: control cells, 3.1; NGF-treated cells, 1.3; VB4T cells, 136. The values presented (mean ± SD of triplicate experiments) represent the difference between total and basal activities.

Characterization of the NGF-induced Decrease of EGF-stimulated Tyrosine Kinase Activity of Differentiated PC12 Cells

As seen in a typical experiment (Fig. 6 A), membranes from cells treated for 3–4 d with NGF show ~60% decrease (Fig. 6 A, lane 3) in the autophosphorylation intensity of the 170,000-D band, when compared with membranes from control cells (Fig. 6 A, lane 2). This is made even more clear by increasing the concentration of the membranes from the treated cells in the phosphorylation assay (Fig. 6 A, lane 4). After alkaline treatment of the gel (Fig. 6 B), the inhibitory effect of NGF treatment on EGF receptor autophosphorylation is even more evident; the levels in untreated cells are no more than 20% of the levels in membranes from control cells. This effect is seen even upon overexposure of the gels (Fig. 6 C). Quantitative analyses of the 170,000-D receptor bands from four different batches of membranes of PC12 cells treated for 4 d with NGF and expressing long neurite processes showed that they incorporated an average of 10 ± 6% as much 32P as did control cell membranes. In another experiment, EGF receptor levels in membranes from control and from NGF-differentiated PC12 cells were measured and compared by assaying both receptor autophosphorylation and src-peptide phosphorylation (Table III). NGF-differentiated cell membranes showed a decrease of 87 and 78% in the tyrosine kinase activity for receptor autophosphorylation and exogenous substrate, respectively.

Possible Mechanisms Involved in the Decrease in EGF Receptor during NGF-induced Differentiation of PC12 Cells

A possible mechanism by which NGF might decrease EGF receptor number is by an activation of cell-mediated receptor internalization. Fig. 7 and Table IV present the results of experiments evaluating the possible difference in the processing of 125I-EGF in control and differentiated cells. The cells were pulsed with 125I-EGF for short periods of time, surface-bound factor removed by a rapid acetic acid wash, and internalized radioactive epidermal growth factor measured (Fig. 7). In addition, internalization and degradation of the bound 125I-EGF was followed by monitoring the overall disappearance of cell-associated radioactivity and the appearance of TCA-precipitable degradation products in the medium (Table IV). The results do not reveal any major difference between control and NGF-treated cells. After 2 h of incubation a parallel process of degradation occurs in both populations (data not shown), resulting after 3 h in 30–40%, and after 9 h in 70% precipitable radioactive degradation products in the medium (Table IV). These studies indicate that the internalization and degradation of the EGF receptor is similar in control and in NGF-treated cells.

Studies by others have shown that some lectins (Vale and Shooter, 1983), a tumor promoter (Boonstra et al., 1985), or certain antibodies (Chandler et al., 1985) can alter the binding properties of EGF receptors in PC12 cells. We have used these agents to probe the binding properties of EGF receptors in control and differentiated cells (Table V). Treatment of the cells at 37°C for 20 min gave the expected inhibitory effect without any major difference between EGF receptors in the two cell populations.
Table V. The Inhibitory Effect of Antibodies, Lectin, Phorbol Ester, and an Ionophore on Specific 125I-EGF Binding to Control and NGF-treated PC12 Cells

|Compound| Concentration| Specific 125I-EGF binding |
|---------|---------------|--------------------------|
|         |               | Control cells | NGF-treated cells |
|         |               | fmol/10^6 cells | fmol/10^6 cells   |
|None     |               |               |                |
|Polyclonal anti-EGF receptor from rat liver| 1:100 dilution of serum| 599 ± 54 | 313 ± 25 |
|151 monoclonal IgG anti-PC12 membranes| 1:1000 dilution of original| 14 ± 2 | 8 ± 3 |
|Wheat germ agglutinin| 25 μg/ml| 8 ± 1 | 6 ± 1 |
|Phorbol 12 myristate 13-acetate| 8 × 10^{-7} M| 138 ± 23 | 120 ± 17 |
|A23187| 10^{-5} M| 388 ± 25 | 227 ± 12 |
|         | 1:1000 dilution of serum| 264 ± 19 | 131 ± 13 |

Cells were preincubated for 20 min in choline buffer to which the indicated concentrations of the various compounds were added. Aliquots of 125I-EGF (0.2 × 10^6 cpm/ml) were then added and incubation was continued for 45 min at 37°C. Values represent average ± SD of triplicate wells.

Since pH-dependent conformational changes in EGF receptors may be important for changes in their affinity or their susceptibility to hydrolysis and cellular processing (DiPaola and Maxfield, 1984), we have compared the pH dependence of EGF binding and its sensitivity to papain hydrolysis in control and differentiated cells (Fig. 8). In both populations of cells, a similar pH dependence profile of EGF binding was measured with an optimum of ~pH 8.0 (Fig. 8 A). In both populations of cells the EGF receptors became resistant to papain proteolysis when the pH was lowered from 7.3 to 5.5 (Fig. 8 B), consistent with the hypothesis that the receptors become less exposed to the external media at low pH (DiPaola and Maxfield, 1984). These observations suggest that EGF receptors from control and from NGF-treated cells undergo overtly similar pH-mediated changes in conformation.

PC12 cells differentiated with NGF exhibit resting membrane potentials of ~50 to ~65 mV (O'Lague and Huttner, 1980), the appearance of voltage-dependent Na⁺ and K⁺ channels (Arner and Stålup, 1981), and Na⁺, K⁺-ATPase (Boonstra et al., 1983), all essentials for their electrical excitability. Because the degree of transmembrane potential in differentiated cells may be a significant factor in determining EGF-receptor properties, we have explored the effect of cell depolarization on 125I-EGF binding to both control and differentiated cells (Table VI). The results indicate that with both populations of PC12 cells, 125I-EGF binding is independent of the transmembrane potential; this potential was significantly affected during the K⁺ depolarization as shown by the reduction in the uptake of the lipophilic permeant cation, 4C-tetraphenylphosphonium bromide (Table VI).

These experiments, together with the 12–24-h lag time required to detect the beginning of the NGF-induced decrease in EGF-binding capacity, distinguish the mechanism of NGF action from previously documented rapid effects of other growth factors on EGF receptor affinity reported in other cells (Rozengurt et al., 1982; Bowen-Pope et al., 1983).

Since the NGF-induced decrease in EGF-binding capacity in differentiated PC12 cells could be related to an increased rate of EGF receptor homodown regulation, we compared the time course of EGF-induced homodown regulation in both populations of cells. In the presence of 10 nM EGF, the binding capacity of both control and differentiated cells was reduced to 50% of its original levels in ~30 min (data not shown). These results indicate that the mechanism of the NGF-induced decrease in EGF-binding capacity in PC12 cells is not related to the homodown regulation process.

NGF-induced Reduction in [35S]methionine Labeling of a 170-kD Glycoprotein Band in the Membranes of Differentiated PC12 Cells

The biosynthesis of the EGF receptor was studied by labeling control and NGF-differentiated PC12 cells with [35S]methionine for 18 h (Mayes and Waterfield, 1984). For comparative purposes A431 cells were labeled under identical conditions. Since a series of monoclonal and polyclonal antibodies directed against the human or rat liver EGF receptor were found ineffective in the immunoprecipitation of PC12 EGF receptors, the radioactive cell membranes were solubilized with detergent and the EGF receptors enriched by wheat germ agglutinin-Sepharose chromatography, a method previously used for the purification of mouse liver EGF receptors (Cohen et al., 1982). Total radioactive glycoproteins bound to the gel were subjected to gel electrophoresis and analyzed by autoradiography. The predominantly labeled proteins bound to wheat germ agglutinin gels have apparent molecular masses of 200,000, 100,000, 95,000, 85,000, and 60,000 D (Fig. 9, lanes b and c). The doublet at 170,000 and 150,000 D was assumed to represent the EGF receptor on the basis of (a) the electrophoretic mobility of [35S]methionine EGF receptors immunoprecipitated from A431 cells (data not shown) and (b) phosphorylation data indicating a single
major autophosphorylated 170,000-D band adsorbed to the wheat germ agglutinin-gels and co-migrating under the same electrophoretic conditions with the 35S-labeled 170,000-D band (Fig. 9, lane d). Similar patterns were seen when N-acetylgalactosamine (300 μM) was used as an elution buffer (data not shown). When the levels of [35S]methionine-labeled EGF receptors in control and NGF-differentiated cell membranes were compared (Fig. 9, lanes b and c), 90 and 50% decreases in the level of the 170,000- and the 150,000-D EGF-receptor bands, respectively, were detected. An almost complete disappearance of both was seen when the carbohydrate eluant was analyzed (data not shown). Examination of the ratio [35S]methionine-labeled 170,000- and 150,000-bands to the 200,000-band (the level of which was found unaffected by the NGF-induced differentiation) in six different batches of control and NGF-differentiated cell membranes indicates 70-90 and 30-50% decrease in the 170,000 and 150,000 receptor species, respectively. Since additional nonreceptor [35S]methionine labeled glycoproteins might be isolated and co-migrate with the receptor bands, these values could be minimum estimates. Consistent with these data is the observation that the intensity of autophosphorylation of the 170,000-D glycoprotein band in differentiated cells (Fig. 9, lane e) showed a >90% decrease compared with control (Fig. 9, lane d), an observation comparable to that made previously with intact membranes. Values represent mean ± SD of triplicate wells.

**Table VI. Effect of Membrane Potential on 125I-EGF Binding and 14C-Tetraphenylphosphonium bromide (TPP) Uptake in Control and NGF-treated PC12 Cells**

| Media               | Specific 125I-EGF binding | 14C]TPP uptake |
|---------------------|---------------------------|---------------|
|                     | Control cells             | NGF-treated cells | Control cells | NGF-treated cells |
|                     | fmol/μg protein           | fmol/μg protein | fmol/μg protein | fmol/μg protein |
| Choline buffer      | 1.9 ± 0.1                 | 0.5 ± 0.1      | 5.0 ± 0.5     | 7.2 ± 0.3       |
| K+ (100 mM) in choline buffer | 2.1 ± 0.1              | 0.5 ± 0.1      | 1.3 ± 1.5     | 3.2 ± 0.2       |

Binding experiments with cell cultures were performed in choline buffer or choline buffer in which choline was replaced by potassium as detailed in Materials and Methods. Membrane potential of the cell cultures was measured using the uptake of [14C]TPP for 60 min at 37°C under normal (choline buffer) and depolarizing (K+/choline buffer) conditions as detailed under Materials and Methods. Uptake values were corrected for radioactivity trapped in the extracellular space. Values represent mean ± SD of triplicate wells.

**Discussion**

In this study we have confirmed our earlier reports indicating that PC12 cells have functional EGF receptors and shown that they express tyrosine kinase activity, a property of these receptors in other cell lines or tissues studied (Hunter and Cooper, 1985). The data presented confirm and further characterize the long-term decrease in EGF-binding capacity of pheochromocytoma cells induced to differentiate by NGF (Huff et al., 1981). This decrease in EGF-binding capacity, originally observed by 125I-EGF binding, is accompanied by a decrease in 170,000- and 150,000-D EGF-receptor species visualized by cross-linking experiments, a decrease in EGF-stimulated receptor autophosphorylation and EGF-stimulated tyrosine kinase activity measured with a src-related peptide as substrate, and a possible decrease in the biosynthesis of the receptor itself as evidenced by a lowered incorporation of [35S]methionine into a 170,000-D glycoprotein band.

The present findings are complemented by the data that show that the decline in the apparent EGF-binding capacity of the differentiated PC12 cells is not due to changes in EGF receptor-binding affinity. Such a change in receptor affinity might have been expected because the physicochemical properties of the plasma membrane of PC12 cells change during differentiation (Guroff, 1985) and also because receptor affinity was found to change during differentiation of mouse neuroblastoma cells (Mummery et al., 1983). In both control and NGF-differentiated PC12 cells, EGF receptors were found to respond similarly to conditions that could affect their affinity, pH-induced conformational changes, and depolarization. Furthermore, no significant differences in internalization, cellular processing of bound EGF, or homodown regulation of the receptor were detected when control and differentiated cells were compared. Indeed, the decrease in EGF-binding capacity of the NGF-differentiated PC12 cells appears to involve a reduction in the
number of cell surface (plasma membrane) receptors, caused by a decreased receptor biosynthesis. Thus, the NGF-induced loss of EGF receptors from PC12 cells fulfills the criteria of a phenomenon of heterologous down regulation (Sibley and Lefkowitz, 1985). Although the short term mechanistic steps responsible for this process need to be elucidated, it is tempting to consider four alternate hypothetical possibilities: (a) in the process of NGF-induced differentiation an EGF-like material or some transforming factor is released to the medium thus affecting EGF receptor occupancy that in the long run leads to decreased receptor biosynthesis; (b) PC12 cells release some glycoproteins into the medium in the process of differentiation (Yavin et al., 1986) that might include EGF receptors, a process shown to occur spontaneously with A431 carcinoma cells (Das et al., 1984); (c) the activation of protein kinase C that was shown recently to occur in PC12 cells in response to NGF (Hama et al., 1986) leads to the phosphorylation of EGF receptors, thus affecting their binding and tyrosine kinase properties as suggested with other cells (Cochet and Hunter, 1984; Fearn and King, 1985), this phosphorylation then serving as a signal for receptor down regulation (Sahyoun et al., 1985) and/or the regulation of receptor biosynthesis (Clark et al., 1985); (d) NGF-induced phosphorylation of a nuclear protein alters EGF receptor synthesis.

The time course of the decrease in EGF receptors in PC12, a 12-24-h lag followed by a gradual loss of receptors for several days, would appear to be the result of some long-term altered rates of receptor synthesis. Studies considering both transcriptional and posttranscriptional processes are underway in an effort to understand the mechanism by which NGF induces the heterodown regulation of EGF receptors during PC12 cell differentiation.

The data in this report and in a previous study (Huff et al., 1981) support a correlation between the loss of EGF receptors and the commitment to NGF-induced differentiation of the PC12 cells, consistent with the earlier observations that NGF inhibits cell division (Greene and Tischler, 1976). One interpretation of these data is that the NGF-induced commitment of proliferating PC12 cells to mature into sympathetic neuron-like cells is correlated temporally with the decrease in EGF receptor levels induced by long-term, progressive heterologous down-regulation. Such differentiation-related changes in EGF-binding capacity are not unique to PC12 cells; similar phenomena were reported in differentiating primary cultures of mouse myoblasts (Lim and Hauschka, 1984) and during spontaneous transformation of Chinese hamster embryo fibroblasts in culture (Wakshull et al., 1985). In contrast, exposure of teratocarcinoma cells to retinoic acid results in the formation of cells with endodermal properties and an increase in EGF-binding capacity and their receptors on chromaffin cells could be important factors in determining the maturation of this organ.

The authors are grateful to Dr. J. Schlessinger (the Weizmann Institute of Science, Rehovot, Israel) for the gift of PK-2 antisera, to Drs. W. A. Dunn and A. L. Hubbard (Johns Hopkins University) for 15I-IgG antibodies, and for discussions held with their colleagues Drs. Joe DeLarco, Nobuo Nakashini, Yuzuru Matsuda, and K. F. Jesse Chan.

Received for publication 4 August 1986, and in revised form 5 February 1987.

References

Adamson, E., and B. L. M. Hogan. 1984. Expression of EGF receptor and transferrin by P9 and PC13 teratocarcinoma cells. Differentiation. 27:152-157.

Arner, L. S., and W. B. Stallcup. 1981. Two types of potassium channels in the PC12 cell line. Brain Res. 215:419-425.

Bocchini, V., and P. U. Angeletti. 1969. The nerve growth factor: purification as a 30,000 molecular weight protein. Proc. Natl. Acad. Sci. USA. 64:787-794.

Boonstra, J., W. H. Moojenaar, P. H. Harrison, P. Moed, P. T. van der Saag, and S. W. de Laat. 1983. Ionic responses and growth stimulation induced by nerve growth factor and epidermal growth factor in rat pheochromocytoma (PC12) cells. J. Cell Biol. 97:92-98.

Boonstra, J., C. L. Mummary, P. T. van der Saag, and S. W. de Laat. 1985. Two receptor classes for epidermal growth factor on pheochromocytoma cells, distinguishable by temperature, lectins and tumor promoters. J. Cell Physiol. 123:347-352.

Bowen-Pope, D. F., P. E. Dicorleto, and R. Ross. 1983. Interactions between the receptors for platelet-derived growth factor and epidermal growth factor. J. Cell Biol. 96:679-683.

Brautigan, D. L., P. Bornstein, and B. Gallis. 1981. Phosphotyrosyl-protein phosphatase. Specific inhibition by Zn²⁺. J. Biol. Chem. 256:6519-6522.

Carpenter, G. 1985. Binding assays for epidermal growth factor. Methods Enzymol. 109:101-110.

Carpenter, G. L., King, Jr., and S. Cohen. 1979. Rapid enhancement of protein phosphorylation in A431 cell membrane preparations by epidermal growth factor. J. Biol. Chem. 254:4884-4891.

Casel, D., and L. Glaser. 1982. Proteolytic cleavage of epidermal growth factor receptor. A Ca²⁺-dependent, sulfhydryl-sensitive proteolytic system in A431 cells. J. Biol. Chem. 257:9845-9848.

Chambaz, L. P., C. E. Chandler, M. Hosang, and E. M. Shooter. 1985. A monoclonal antibody which inhibits epidermal growth factor binding has opposite effects on the biological action of epidermal growth factor in different cell types. J. Biol. Chem. 260:3360-3367.

Clark, A. J. L., S. Ishii, N. Richert, G. T. Merlino, and I. Pastan. 1985. Epidermal growth factor regulates the expression of its own receptor. Proc. Natl. Acad. Sci. USA. 82:8376-8378.

Cochet, C., G. N. Gill, J. Meisenhelder, J. A. Cooper, and T. Hunter. 1984. C-kinase phosphorylates the epidermal growth factor receptor and reduces its epidermal growth factor-stimulated tyrosine protein kinase activity. J. Biol. Chem. 259:2553-2558.

Cohen, S. 1983. Purification of the receptor for epidermal growth factor from A431 cells: its function as a tyrosyl kinase. Methods Enzymol. 99:379-387.

Cohen, S., R. A. Fava, and S. T. Sawyer. 1982. Preparation of the epidermal growth factor receptor/protein kinase from normal mouse liver. Proc. Natl. Acad. Sci. USA. 79:6237-6241.

Cooper, J. A., B. M. Sefton, and T. Hunter. 1983. Detection and quantification of phosphorysine in proteins. Methods Enzymol. 99:387-402.
Das, M., R. Biswas, M. Basu, and S. Bishayee. 1984. Mitogenic activity of the epidermal growth factor receptor after transfer from A431 carcinoma cells to receptor-negative fibroblast cells. *Cancer Res.* 44:3539-3545.

DiPaola, M., and F. R. Maxfield. 1984. Conformational changes in the receptors for epidermal growth factor and asialoglycoproteins induced by the mildly acidic pH found in the endocytic vesicles. *J. Biol. Chem.* 259:9163-9171.

Dunn, W. A., T. P. Connolly, and A. L. Hubbard. 1986. Receptor-mediated endocytosis of epidermal growth factor by rat hepatocytes: receptor pathway. *J. Cell Biol.* 102:24-36.

Fain, R. A., and G. Guroff. 1980. Isolation of a calcium-dependent 35 kilodalton substrate for the epidermal growth factor receptor/kinase from A431 cells. *J. Biol. Chem.* 255:2636-2645.

Fearn, J. C., and A. C. King. 1985. EGF receptor affinity is regulated by intracellular calcium and protein kinase C. *Cell.* 40:991-1000.

Greene, L. A., and A. S. Tischler. 1976. Establishment of a noradrenergic clonal line of rat adrenal pheochromocytoma cells which respond to nerve growth factor. *Proc. Natl. Acad. Sci. USA.* 73:2785-2789.

Guroff, G. 1983. Nerve growth factor and other factors acting on the nervous system. In *Handbook of Neurochemistry.* Vol. 5. 2nd ed. A. Lajtha, editor. Plenum Publishing Corp., New York. 443-466.

Guroff, G. 1985. PC12 cells as a model of neuronal differentiation. In *Cell Culture in the Neurosciences.* J. E. Botstein and G. Sato, editors. Plenum Publishing Corp., New York. 245-271.

Haigler, H. T., M. C. Willingham, and I. Pastan. 1980. Inhibitors of 125I-EGF internalization. *Biochem. Biophys. Res. Commun.* 94:630-637.

Hama, T., K. P. Huang, and G. Guroff. 1986. Protein kinase C as a component of a nerve growth factor-sensitive phosphorylation system in PC12 cells. *Proc. Natl. Acad. Sci. USA.* 83:2353-2357.

Herrick, K., and H. Thoenen. 1979. Properties of the nerve growth factor receptor of a clonal line of rat pheochromocytoma (PC12) cells. *Exp. Cell Res.* 121:71-78.

Hollenberg, M. D., and P. Cuatrecasas. 1975. Insulin and epidermal growth factor: human fibroblast receptors related to deoxyribonucleic acid synthesis and amino acid uptake. *J. Biol. Chem.* 250:3845-3853.

Huff, K., and G. Guroff. 1979. Nerve growth factor-induced reduction of epidermal growth factor responsiveness and epidermal growth factor receptors in PC12 cells: an aspect of cell differentiation. *Biochem. Biophys. Res. Commun.* 89:175-180.

Huff, K., D. End, and G. Guroff. 1981. Nerve growth factor-induced alteration in the response of PC12 pheochromocytoma cells to epidermal growth factor. *J. Cell Biol.* 88:189-198.

Hunter, T., and J. Cooper. 1983. Protein-tyrosine kinases. *Annu. Rev. Biochem.* 52:71-102.

Johnson, L. K., J. D. Baxter, I. Vlodavsky, and D. Gospodarowicz. 1980. EGF receptor affinity is regulated by intracellular calcium and protein kinase C. *Cell.* 40:991-1000.

Kris, R. M., I. Lax, W. Gullick, M. D. Waterfield, A. Ullrich, M. Fridkin, and J. Yarden. 1981. Nerve cells in clonal systems. *Nature (Lond.)* 287:739-742.

Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature (Lond.)* 227:809-827.

Landreth, G. E., and E. M. Shooter. 1980. Nerve growth factor receptors on PC12 cells: ligand-induced conversion from low to high affinity states. *Proc. Natl. Acad. Sci. USA.* 77:4751-4755.

Layer, P. G., and E. M. Shooter. 1982. Binding and degradation of nerve growth factor by PC12 pheochromocytoma cells. *J. Biol. Chem.* 258:3012-3018.

Lim, R. W., and S. D. Hauschka. 1984. A rapid decrease in epidermal growth factor binding capacity accompanies the terminal differentiation of mouse myoblasts in vitro. *J. Cell Biol.* 96:739-742.

Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall. 1951. Protein measurement with the folin phenol reagent. *J. Biol. Chem.* 193:265-275.

Matsrin, L. M., B. R. Larsen, J. S. Finch, and B. E. Magun. 1982. Further purification of epidermal growth factor by high performance liquid chromatography. *Anal. Biochem.* 125:339-351.

Mayes, E. L. V., and M. D. Waterfield. 1984. Biosynthesis of the epidermal growth factor receptor in A431 cells. *EMBO (Eur. Mol. Biochem. Organ.)* 3:531-537.

Milligan, G., and P. G. Strange. 1984. Use of 125I-epidermal growth factor cation for estimating membrane potential in neuroblastoma cells. *J. Neurochem.* 43:1515-1521.

Mummery, C. L., P. T. van Saag, and S. W. de la Laat. 1983. Loss of EGF binding and cation transport response during differentiation of mouse neuroblastoma cells. *J. Cell. Biochem.* 21:63-75.

O'Lague, P. H., and S. L. Hutner. 1980. Physiological and morphological studies of rat pheochromocytoma cells (PC12) chemically fused and grown in culture. *Proc. Natl. Acad. Sci. USA.* 77:1701-1705.

Petrides, P. E., and E. M. Shooter. 1986. Rapid isolation of the 7S nerve growth factor complex and its subunits from murine submaxillary glands and salivary. *J. Neurochem.* 46:721-725.

Pike, L. J., B. Gallis, J. E. Casanelli, P. Bornstein, and E. G. Krebs. 1982. Epidermal growth factor stimulates the phosphorylation of synthetic tyrosine-containing peptides by A431 cell membranes. *Proc. Natl. Acad. Sci. USA.* 79:1443-1447.

Rozengurt, E., M. Collins, K. D. Brown, and P. Pettic. 1982. Inhibition of epidermal growth factor binding to mouse cultured cells by fibroblast-derived growth factor. *J. Biol. Chem.* 257:3680-3686.

Sahyoun, W. S. M. Jr., N. M. Wolf, and P. Cuatrecasas. 1985. Role of intracellular calcium mobilization in the regulation of protein kinase C-mediated membrane processes. *Nature.* 317:549-551.

Savage, C. R., Jr., and S. Cohen. 1972. Epidermal growth factor and a new derivative. *J. Biol. Chem.* 247:7609-7611.

Schlossberg, M., J. B. Bliezizkan, M. Apfelbaum, and R. C. Bennett. 1978. 125-Adrenergic receptors and myogenesis. *J. Cyclic Nucleotide Res.* 4:55-69.

Schwartz, K. R., S. M. Lanier, A. E. Carter, J. H. Honcy, and R. M. Graham. 1985. Rapid reciprocal changes in adrenergic receptors in intact isolated hepatocytes during primary cell culture. *Mol. Pharmacol.* 27:200-209.

Sibley, D. R., and R. J. Lefkowitz. 1985. Molecular mechanisms of receptor desensitization using the 125-Adrenergic receptor-coupled adenylate cyclase system as a model. *Nature.* 317:124-129.

Swank, L. R., S. Cohen, and D. L. Garbers. 1982. Inhibition of membrane phospho-tyrosyl-protein phosphatase activity by vanadate. *Biochem. Biophys. Res. Commun.* 107:1104-1109.

Togari, A., G. Dickens, H. Kuzaya, and G. Guroff. 1985. The effect of fibroblast growth factor on PC12 cells. *J. Neurosci.* 5:307-316.

Tower, B. B., R. C. Clark, and R. T. Rubin. 1977. Preparation of 125I poly peptide hormones for radioimmunoassay using glucose oxidase and laccase oxidoreductase. *Life Sci.* 21:959-961.

Tuszynski, G. P., L. Knight, J. R. Piperno, and P. N. Walsh. 1980. A rapid method for removal of [125I] iodide following iodination of protein solutions. *Anal. Biochem.* 106:118-122.

Vale, R. D., and E. M. Shooter. 1983. Epidermal growth factor receptors on PC12 cells: alteration of binding properties by lectins. *J. Cell. Biochem.* 22:99-109.

Wakshull, E., P. M. Kraemer, and W. Wharton. 1985. Multistep change in epidermal growth factor receptors during spontaneous neoplastic progression in Chinese hamster embryo fibroblasts. *Cancer Res.* 45:2070-2075.

Yavin, E., T. Hama, S. Gil, and G. Guroff. 1986. Nerve growth factor and gangliosides stimulate the release of glycoproteins from PC12 pheochromocytoma cells. *J. Neurochem.* 46:794-803.