Down-regulation of Epidermal Growth Factor Receptors by Nerve Growth Factor in PC12 Cells Is p140trk-, Ras-, and Src-dependent

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Nerve growth factor (NGF) treatment causes a profound down-regulation of epidermal growth factor receptors during the differentiation of PC12 cells. This process is characterized by a progressive decrease in epidermal growth factor (EGF) receptor level measured by 125I-EGF binding, tyrosine phosphorylation, and Western blotting. Treatment of the cells with NGF for 5 days produces a 95% reduction in the amount of 125I-methionine-labeled EGF receptors. This down-regulation does not occur in PC12nnr5 cells, which lack the p140trk NGF receptor. However, in PC12nnr5 cells stably transfected with p140trk, the NGF-induced heterologous down-regulation of EGF receptors is reconstituted in part. NGF-induced heterologous down-regulation, but not EGF-induced homologous down-regulation of EGF receptors, is blocked in Ras- and Src-dominant-negative PC12 cells. Treatment with either pituitary adenylate cyclase-activating peptide (PACAP) or staurosporine stimulates neurite outgrowth in PC12 cell variants, but neither induces down-regulation of EGF receptors. NGF treatment of PC12 cells in suspension induces down-regulation of EGF receptors in the absence of neurite outgrowth. These results strongly suggest a p140trk-, Ras- and Src-dependent mechanism of NGF-induced down-regulation of EGF receptors and separate this process from NGF-induced neurite outgrowth in PC12 cells.

PC12 cells, derived from a rat pheochromocytoma, have been extensively used as a model of neuronal differentiation (1), because the cells acquire the phenotype of sympathetic neurons and stop dividing in response to nerve growth factor (NGF). In PC12 cells, NGF interacts with two distinct plasma membrane receptor proteins: p75NGFR, a cysteine-rich glycoprotein having a relatively low affinity for NGF (3), and p140trk, a receptor tyrosine kinase activated by NGF, which binds NGF with high affinity and mediates many of the biological activities of this neurotrophin (4). The binding of NGF to the p140trk receptor stimulates rapid tyrosine autophosphorylation of the receptor and activation of several signal-transducing proteins (4). Activated p140trk receptors bind to and tyrosine phosphorylate the signaling substrates phospholipase Cγ1, phosphatidylinositol-3 kinase, and Src homologous collagen protein (4), resulting in their activation. The latter stimulates Ras activity which subsequently activates a series of serine/threonine kinases including B-Raf, myelin basic protein kinase kinase, the Erks, and p90rsk (4). This Ras-Erk pathway plays a major role in the activation of transcriptional events by NGF and in NGF-induced neuronal differentiation (5), as illustrated by inhibition of NGF actions upon expression of dominant-negative Ha-Ras (Asn-17) proteins (6, 7). In addition a role for pp60src in NGF actions has been suggested (5) by experiments involving the expression of a dominant-interfering kinase-inactive Src (8).

PC12 cells also express plasma membrane receptors for epidermal growth factor (EGF) (9, 10), a mild mitogen for these cells (9). PC12 cells treated with NGF exhibit an attenuated response to EGF (9) because EGF receptors are down-regulated (9, 11). The cellular mechanisms that mediate NGF-induced EGF receptor down-regulation are unknown and represent an interesting question in neuronal development. The existence of functional receptors for both NGF and EGF on PC12 cells and the recent advances in understanding the signaling pathways activated by these receptors make these cells a useful model for the study of cross-regulation between NGF and EGF receptors during differentiation.

In this study, we have used a number of PC12 variant cell lines with different levels of p140trk (12, 13) and EGF receptor expression and dominant-negative Ras (7, 14, 15) or Src (5, 6, 8) PC12 transfectants in an attempt to elucidate the molecular mechanisms of the receptor cross-talk required by NGF to down-regulate EGF receptors. Our findings implicate the involvement of p140trk-, Ras-, and Src-dependent signaling pathways in NGF-induced down-regulation of EGF receptors while

K-252a, 8R*,9S*,11S*,-9-hydroxy-9-methoxycarbonyl-8-methyl-2,3,9,10-tetrayahdro-8,11-epoxy-1H,7H,11H-2,7,11-triazafluorenone; B-Raf, 11a-triazafluorenone (9); 8(R*,9S*,11S*)-(-)-9-hydroxy-9-methoxycarbonyl-8-methyl-2,3,9,10-tetrayahdro-8,11-epoxy-1H,7H,11H-2,7,11-triazafluorenone; Myc, myc proliferator; MBP, myelin basic protein; PAG, polyacrylamide gel electrophoresis; DMEM, Dulbecco's modified Eagle's medium; FGF, fibroblast growth factor.

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The abbreviations used are: NGF, nerve growth factor; 6,24, a pheochromocytoma clone overexpressing human p140trk; PC12nnr5, a pheochromocytoma clone nonresponsive to nerve growth factor; GSraDN6, dexamethasone-inducible, dominant-negative Ras PC12 variant cell line; M-M17-26, stable, dominant-negative Ras PC12 variant cell line; SrcDN2, stable, dominant-negative Src PC12 variant cell line; p140trk, high affinity nerve growth factor receptor; EGF, epidermal growth factor; PACAP, pituitary adenylate cyclase-activating peptide;
demonstrating an independence of this cellular process from NGF-induced neurite outgrowth.

EXPERIMENTAL PROCEDURES

Materials—Mouse NGF, EGF, bovine brain-derived acidic FGF and basic FGF, and rat collagen type II were purchased from Collaborative Biochemicals (Bedford, MA). Dexamethasone, poly-L-lysine, and myelin basic FGF, and rat collagen type II were purchased from Collaborative Biosciences (Bedford, MA). The medium was changed, and fresh NGF was added every other day. Binding of $^{125}$I-EGF ($0.2 \times 10^5$ cpm/1 $\times 10^5$ cells) was measured by incubation for 45 min at 37 °C in the presence or the absence of excess unlabeled EGF (10 $-6$ M). The error bars indicate the standard deviation. All points differ from 0 day with a p value of <0.05. The upper panel shows control PC12 cells and the typical neurite outgrowth induced by NGF after 76 and 120 h of treatment. The panel inset shows the Western blots of PC12 cell lysates obtained from control cultures (C) and cultures treated with 50 ng/ml NGF for 2 days (2d), 4 days (4d), and 6 days (6d) using the 1210 polyclonal anti-EGF receptor (arrow, 170 kDa) antibody.

FIG. 1. Time course of NGF-induced down-regulation of EGF receptors in PC12 cells. PC12 cell cultures were grown in medium with 50 ng/ml NGF. The medium was changed, and fresh NGF was added every other day. Binding of $^{125}$I-EGF ($0.2 \times 10^5$ cpm/1 $\times 10^5$ cells) was measured by incubation for 45 min at 37 °C in the presence or the absence of excess unlabeled EGF (10 $-6$ M). The error bars indicate the standard deviation. All points differ from 0 day with a p value of <0.05. The upper panel shows control PC12 cells and the typical neurite outgrowth induced by NGF after 76 and 120 h of treatment. The panel inset shows the Western blots of PC12 cell lysates obtained from control cultures (C) and cultures treated with 50 ng/ml NGF for 2 days (2d), 4 days (4d), and 6 days (6d) using the 1210 polyclonal anti-EGF receptor (arrow, 170 kDa) antibody.

NGF-induced Down-regulation of EGF Receptors in PC12 Cells

were grown in collagen-coated tissue culture dishes in RPMI 1640 medium (Life Technologies, Inc.) supplemented with 10% horse serum and 5% fetal bovine serum (13). 6.24 cells, a clone of PC12 overexpressing human p140$^{+A}$, were grown under the same conditions as PC12 cells in the presence of 200 µg/ml G418 (Life Technologies, Inc.) (12). During the down-regulation experiments, G418 was removed from the medium. The PC12 cell variants, G8rasDN6 expressing a dominant-negative mutant ras gene (15) under the transcriptional control of the mouse mammary tumour virus promoter, M-M17-26 expressing the Ha-ras Aas-17 gene under the transcriptional control of the mouse metallothionein-I promoter (7), and srcDN2 expressing the K295R mutant (kinase dead) form of chicken Src under the control of the cytomegalovirus promoter (8) were grown, as were PC12 cells, in DMEM supplemented with 10% fetal bovine serum and 5% horse serum. In a series of experiments involving dexamethasone treatment, the cells were placed in charcoal-treated serum 24 h before stimulation with 500 nM dexamethasone. To grow cells in suspension, PC12 cells were dispersed in 50 ml of complete medium at a density of 1 $\times 10^5$ cells/ml in a 250-ml polycarbonate spinner flask (Corning) (18). The suspension was gently shaken at 37 °C on a rotary shaker in an incubator.

Binding Assays—Radioreceptor assays were carried out as described previously (11). The cells were grown on collagen (200 µg/ml)- and polylysine (10 µg/ml)-coated six-well plates (Costar). Cell culture monolayers were washed and equilibrated with fresh, serum-supplemented DMEM for 1 h. Total binding of growth factors was measured by adding saturating concentrations of $^{125}$I-EGF (0.5 $\times 10^6$ cpm/ml) or 50–100 pM $^{125}$I-NGF (to measure high affinity p140$^{+A}$ receptors). Nonspecific binding was evaluated by adding a 100-fold excess of unlabeled growth factors to sister cultures and was typically 5–10% and 20–30% of total binding for EGF and NGF, respectively. The incubation, unless otherwise stated, was carried out at 37 °C for 45 min, then the incubation buffer was removed, and the cells were washed with ice-cold phosphate-
binding of 125I-EGF was measured as described under "Experimental Procedures." The error bars indicate the S.D. NGF differs from control with a p value of <0.01 and in B with a p value of <0.05.

buffered saline (138 mM NaCl, 2.7 mM KCl, 8 mM NaHPO₄, 1.5 mM KH₂PO₄). The cell monolayer was solubilized with 1 ml of 1 N NaOH overnight at room temperature. The cell-associated radioactivity was counted in a γ-counter; a portion of the solubilized mixture was used for protein determination. Data points represent specific binding values and are the means of quadruplicate samples minus the nonspecific binding, expressed as counts/min/mg of protein (NGF) or counts/min/mg of protein (NGF) ± S.D. Binding experiments with cells grown in suspension were performed for 45 min in suspension, using 3 × 10⁶ cells/ml of medium with ¹²⁵I-EGF (1 × 10⁴ cpm/ml) in the presence or absence of 10 µg/ml unlabeled EGF. The separation of free ¹²⁵I-EGF was measured as described under Experimental Procedures. The specific binding of ¹²⁵I-EGF was measured as described under Experimental Procedures. The specific binding of ¹²⁵I-EGF was measured as described under "Experimental Procedures." The error bars indicate the S.D. NGF differs from control with a p value of <0.01 and in B with a p value of <0.05.

the radioreceptor assay was performed in 200 µl of binding buffer (DMEM containing 10 µM heparin, 0.2% bovine serum albumin, 25 mM HEPES, pH 7.4) with 0.4 ng of either ¹²⁵I-labeled acidic FGF or ¹²⁵I-labeled basic FGF in the presence or absence of 50 ng of the respective unlabeled growth factor, as described previously (17). Binding was terminated with two washes with binding buffer followed by a third wash for 5 min with binding buffer at pH 7.5 or pH 4.0 for low and high affinity FGF binding, respectively (17).

**Cell Labeling**—For iodination of cell surface proteins, 3 × 10⁶ cells grown on 10-cm collagen- and polylysine-coated tissue culture dishes were incubated with 1 µCi of Na¹²⁵I and 0.2 mg/ml lactoperoxidase (19). In other experiments, PC12 cell cultures were incubated for 18 h in methionine-free DMEM containing 100 µCi/ml [³⁵S]methionine/cysteine (Translabel; ICN, Plainview, NY). For immunoprecipitation of EGF receptors, the cell monolayers were washed twice with DMEM and solubilized for 30 min at 4 °C in radiolabeled precipitation buffer (50 mM Tris-HCl, pH 7.4, 0.15 M NaCl, 1% Triton X-100, 0.1% SDS, 1% deoxycholate, 1 mM phenylmethylsulfonyl fluoride, 1 mg/ml aprontin) (11). Insoluble material was removed by centrifugation, and solubilized material was subjected to immunoprecipitation using anti-EGF receptor antibody.

**Immunoprecipitation and Immunoblotting**—Cells were plated in 10-cm tissue culture dishes 1 day prior to the experiment. Following treatment with growth factors, cells were washed twice with ice-cold Tris-buffered saline (20 mM Tris-HCl, pH 8.0, 137 mM NaCl) and subjected to lysis in 1 ml of 1% Nonidet P-40 in lysis buffer (20 mM Tris-HCl, 137 mM NaCl, 0.5 mM EDTA, 10% glycerol, 1 mM phenylmethylsulfonyl fluoride, 0.15 unit/ml aprotinin, 20 mM leupeptin, 1 mM sodium vanadate) at 4 °C for 20 min. Insoluble material was removed by centrifugation and solubilized material was subjected to immunoprecipitation with anti-trkA or anti-Erk antibodies for 2 h at 4 °C with continuous agitation, followed by an additional 2-h incubation with protein A-Sepharose. Precipitates were washed three times with lysis buffer and once with water, then boiled for 5 min in SDS sample buffer (0.06 mM Tris-HCl, pH 6.8, 12.5% glycerol, 1.25% SDS, 5% β-mercaptoethanol, 0.002% bromphenol blue), and subjected to SDS-PAGE on 7.5% polyacrylamide gels, followed by transfer to nitrilemembrane. Blots were probed overnight at 4 °C with the primary antibodies and analyzed using an enhanced or Tropix chemiluminescence system and horseradish peroxidase-coupled secondary antibodies and quantitated by densitometry.

**Kinase Assays**—EGF-dependent tyrosine kinase activity was assayed in both whole cells and cell-free membrane fractions. PC12 cells were labeled for 4 h with [³²P]orthophosphate (0.25 µCi/ml) in phosphate-free DMEM (16). For membrane experiments, 200 µg of PC12 cell membranes were preincubated with EGF (10 ng/ml) for 15 min at 37 °C in a reaction buffer containing 20 mM HEPES, pH 7.2, 0.5 mM MgCl₂, 3 mM MnCl₂, and 50 µM NaVO₄ in a final volume of 50 µl (13). The reaction was initiated by the addition of 2 µCi of [γ-³²P]ATP in 50 µM unlabeled ATP for 5 min at 4 °C. The reaction was terminated by the

**TABLE I**

| Treatment | Acidic FGF | Basic FGF |
|-----------|------------|-----------|
|           | High       | Low       | High       | Low       |
| Control   | 6,187 ± 200 | 7,287 ± 100 | 13,869 ± 250 | 11,065 ± 300 |
| NGF       | 6,760 ± 120 | 6,586 ± 120 | 12,396 ± 400 | 13,313 ± 460 |

**FIG. 2.** Effect of PACAP and staurosporine on neurite outgrowth and ¹²⁵I-EGF binding in PC12 cells. A, PC12 cells were treated for 4 days with either 50 ng/ml NGF (c), 20 nM PACAP (d), or left untreated (b). B, PC12 cells were treated for 60 h with either 50 ng/ml NGF (c), 50 nM staurosporine (d), or left untreated (b). The specific binding of ¹²⁵I-EGF was measured as described under Experimental Procedures. The error bars indicate the S.D. NGF differs from control in A with a p value <0.01 and in B with a p value of <0.05.

Un-treated PC12 cells and PC12 cells treated for 7 days with NGF (50 ng/ml) were grown in 24-well plates coated with collagen/ polylysine. The medium was changed, and NGF was added every other day. Then the cells were washed twice with DMEM containing 20 mM HEPES, pH 7.5, and 0.1% bovine serum albumin. The cultures were incubated for 2 h at 4°C with 2 ng/ml of either ¹²⁵I-labeled acidic or basic FGF in the same medium supplemented with 200 ng/ml heparin. The binding was stopped by washing the cells with fresh binding medium. To dissociate low affinity bound factor the cultures were incubated for 5 min in phosphate-buffered saline, pH 7.5, containing 25 mM HEPES and 2 mM NaCl; to dissociate high affinity bound factor, the cultures were then incubated for another 5 min in phosphate-buffered saline, pH 4.0, containing 20 mM HEPES and 2 mM NaCl (19). Nonspecific binding was measured in the presence of 200 ng/ml of the appropriate unlabeled FGF. The values represent the mean ± S.D. of three different experiments each performed in duplicate.
NGF-induced Down-regulation of EGF Receptors in PC12 Cells

Characteristics of NGF-induced down-regulation of EGF receptors in PC12 cells. A, NGF-induced down-regulation of EGF receptors as measured by binding assays with plasma membranes from PC12 cells treated with NGF for the indicated times. The error bars indicate the S.D. Inset, Western blotting with 1210 anti-EGF receptor antibody of 40 μg of membrane protein samples of control (C) and cells treated for 72 h with NGF. B, expression level of EGF receptors on control (C) and NGF-treated (5 days; 50 ng/ml) PC12 cells. PC12 cell cultures were iodinated as described under "Experimental Procedures," and 0.7 mg of each lysate was immunoprecipitated with 1210 anti-EGF receptor antibody (50 μg/ml). The immunoprecipitates were analyzed by 7.5% SDS-PAGE, and the resulting autoradiogram is presented. The positions of molecular mass markers are indicated on the left. C, untreated (C) and NGF-treated (7 days; 50 ng/ml) PC12 cells were labeled with [32P]orthophosphate (0.1 μCi/ml) for 4 h and incubated with EGF (10 ng/ml) for 10 min (+). Cell lysates (5 mg) were immunoprecipitated with 1210 anti-EGF receptor antibody (50 μg/ml), half the sample was incubated with [γ32P]ATP (left side), and the other half was resolved on 7.5% SDS-PAGE, immunoblotted with anti-phosphotyrosine antibody (1:10,000 dilution), and visualized by chemiluminescence (right side). E, analysis of [35S]methionine incorporation into EGF receptors in control (C) and NGF-treated (50 ng/ml; 7 days) PC12 cells. Cell lysates (5 mg) were immunoprecipitated with 1210 anti-EGF receptor antibody (50 μg/ml), the immunoprecipitates were resolved on 7.5% SDS-PAGE, and the resulting autoradiogram is presented. Both the intact 170-kDa and the proteolytically derived 150-kDa EGF receptor (EGFR) proteins are shown.

Results

NGF-induced Down-regulation of EGF Receptors—The level of 125I-EGF binding during NGF-induced differentiation of PC12 cells was examined (Fig. 1). Treatment of the cells with 50 ng/ml NGF progressively reduced the level of 125I-EGF binding; 50% reduction was seen after 3 days of treatment. In wild-type PC12 cells, this decrease in binding represents a decrease in EGF receptor level (Fig. 1, top). The neuropeptide PACAP induces similar, but somewhat less robust neurite outgrowth in PC12 cells, but does not affect the binding of 125I-EGF (Fig. 2A). Staurosporine, a microbial protein kinase inhibitor, also induces rapid neurite outgrowth in PC12 cells, but, in contrast to NGF, increases 125I-EGF binding up to 3-fold (Fig. 2B). By way of comparison, NGF-induced differentiation of PC12 cells does not affect the level of either high or low affinity receptors for acidic or basic FGF (Table I) or of receptors for insulin-like growth factor I (20).
NGF-induced Down-regulation of EGF Receptors in PC12 Cells

NGF-induced down-regulation of EGF receptors in PC12 cells can also be measured in isolated plasma membrane fractions derived from cells treated with NGF (Fig. 3A). Iodination of EGF receptors on PC12 cell membranes with lactoperoxidase and Na\(^{125}\)I in control and 5-day NGF-treated cells, followed by immunoprecipitation of the labeled EGF receptors indicated a 98% decrease in receptor level (Fig. 3B). Another criterion of NGF-induced, down-regulation of EGF receptors would be a decrease in EGF-stimulated tyrosine kinase activity of EGF receptors. In both whole cells (Fig. 3C) and membrane fractions (Fig. 3D), phosphorylation of EGF receptors was very low or absent after 7 days of NGF treatment. When the levels of \[^{35}\text{S}\]\text{methionine-labeled, immunoprecipitated EGF receptors in absence after 7 days of NGF treatment. When the levels of NGF and EGF receptors in these several PC12 cell variants were compared (Fig. 7), 60 and 95% decreases in the level of 170- and 150-kDa EGF receptor proteins, respectively, were seen.

NGF-induced Down-regulation of EGF Receptors Is \(p_{140}^{trk}\)-dependent—PC12 cells express two types of receptors for NGF: the high affinity receptor \(p_{140}^{trk}\) and the low affinity receptor \(p_{75}^{NGFR}\) (3). In PC12nnr5 cells, a PC12 variant that expresses \(p_{75}^{NGFR}\), but very little \(p_{140}^{trk}\) (13), NGF does not induce neurite outgrowth. When human \(p_{140}^{trk}\) was transfected into PC12nnr5 cells, they grow neurites in response to NGF stimulation (21). Also, overexpression of \(p_{140}^{trk}\) in wild-type PC12 cells accelerated NGF-induced neurite outgrowth in the 6.24 PC12 cell variant (12).

To determine if NGF-induced down-regulation of EGF receptors is mediated by \(p_{140}^{trk}\) receptors, we first estimated the levels of NGF and EGF receptors in these several PC12 cell variants (Fig. 4). Indeed, compared with wild-type PC12 cells, PC12nnr5 cells express very low levels of \(p_{140}^{trk}\) receptors, but had 63% of the EGF receptors seen in the wild-type PC12 cells, while the 6.24 cell variant expressed 240 and 205% more \(p_{140}^{trk}\) and EGF receptors, respectively, than did the wild-type. The level of NGF receptors in PC12nnr5 cells was not altered, even after 7 days of NGF treatment as evaluated by binding (Fig. 5). However, the EGF receptors in these cells could be rapidly homologously down-regulated by exposure of the cells to EGF. This is a reversible process, as indicated by the reappearance of cell surface binding upon removal of EGF (Fig. 5).

PC12nnr5 cells were stably transfected with human \(p_{140}^{trk}\) as recently described (21). Out of 120 clones generated, a dozen were selected based on their expression of \(p_{140}^{trk}\) and EGF receptors and the ability of NGF treatment to down-regulate the EGF receptors. As indicated in Table II, four clones were further characterized. Clone 42 is a \(p_{140}^{trk}\) overexpressor, clones 61 and 106 have levels of \(p_{140}^{trk}\) similar to those in PC12 cells, and clone 50 expresses much lower levels of \(p_{140}^{trk}\) (Table II). These binding data are supported by the Western blots presented in Fig. 6, indicating different degrees of EGF receptor down-regulation in the four clones. This figure also shows that the transfected trk receptors are functional in that they mediate the stimulation of Erk phosphorylation by \(50 \text{ ng/ml NGF}\) (Fig. 6). The NGF-induced down-regulation of EGF receptors was measured in all four clones. No direct correlation was found between NGF-induced neurite outgrowth and NGF-induced down-regulation of EGF receptors (Table II). NGF does not down-regulate EGF receptors in PC12nnr5 cells, but does so (30–60% down-regulation) after introduction of \(p_{140}^{trk}\) into these cells (Table II). Therefore, it seems likely that NGF-induced down-regulation is mediated by \(p_{140}^{trk}\). Support for this suggestion was also provided by experiments with K-252a (Fig. 7). K-252a, a specific inhibitor of NGF-induced \(p_{140}^{trk}\) tyrosine kinase activity in whole PC12 cells (22), prevented the down-regulation of EGF receptors by NGF in both parental PC12 cells (Fig. 7A) and 6.24 PC12 \(p_{140}^{trk}\)-overexpressing cells (Fig. 7B). Although the NGF-induced neurite outgrowth in 6.24 cells is accelerated as it is in PC12nnr5-p\(_{140}^{trk}\)-42 cells, the time course of NGF-induced down-regulation of EGF receptors is similar to that in wild-type PC12 cells (Fig. 1), reaching 50% decrease in receptor levels in about 48–72 h (Fig. 7B).

NGF-induced Down-regulation of EGF Receptors is Ras-dependent—To test whether NGF-induced down-regulation of EGF receptors is dependent upon Ras activation, two PC12 cell variants, transiently (GSrasDN6) or stably (M-M17-26) over-expressing the dominant-negative mutant Ras\(^{N17}\) were employed. As shown in Fig. 4, these variants express different...
levels of p140

Expression of the dominant-negative Ras protein is induced in GSrasDN6 and increased in M-M17-26 by treatment with dexamethasone (14, 15). To determine whether the dominant-negative Ras protein affects the stimulation of Erks under the conditions in which NGF-induced down-regulation was measured, we examined NGF- or EGFinduced phosphorylation of MBP by immunoprecipitated Erks in stably transfected GSrasDN6 cells and NGF-induced phosphorylation of MBP by immunoprecipitated Erks in M-M17-26 cells compared with wild-type PC12 cells (Fig. 8C). In GSrasDN6 cells not treated with dexamethasone, NGF and EGFinduced a 12- and 11-fold increase in Erk1 activity, respectively, measured by the phosphorylation of myelin basic protein (Fig. 8C). In dexamethasone-treated GSrasDN6 cells, the NGF- or EGFinduced increases in Erk1 activity were almost completely inhibited (Fig. 8C). Under identical conditions, a 25% reduction in 125I-EGF binding could be measured after 48 h of NGF treatment (Fig. 8A) or 4–5 days (data not shown) in GSrasDN6 cells not treated with dexamethasone (Fig. 8A). However, a complete homologous down-regulation of EGFinduced phosphorylation could be observed (Fig. 8A). Upon dexamethasone treatment, the small NGF-induced down-regulation effect was blocked, but not the EGFinduced homologous down-regulation of EGFinduced phosphorylation (Fig. 8A).

In M-M17-26 cells in which the dominant-negative RasN17 is stably overexpressed, neither NGF nor EGFinduced Erk1-induced MBP phosphorylation (Fig. 8C). In these cells, NGF-induced heterologous down-regulation of EGFinduced phosphorylation is completely blocked (Fig. 8B), but the EGFinduced homologous

### Table II

| Clone | NGF-induced neurite outgrowth | 125I-NGF binding | 125I-EGF binding |
|-------|-------------------------------|------------------|------------------|
|       |                               | Control          | NGF              |
|       |                               | cpm/mg protein   | cpm/mg protein   |
| 42    | Very fast (6–10 h), extensive sprouting, long, branched neurites | 10,950 ± 1860 | 8.2 ± 0.6 |
| 50    | Delayed appearance (24 h), short neurites | 1,750 ± 311 | 12.0 ± 0.8 |
| 61    | Delayed appearance (24 h), extensive sprouting | 4,125 ± 330 | 6.4 ± 0.6 |
| 106   | Very fast (4–6 h), extensive sprouting of very long neurites | 3,382 ± 642 | 24.1 ± 4.2 |

*These clones were propagated for 3–4 months in culture and showed stable expression of p140

The percent of cells bearing neurites and the length of neurite outgrowth was estimated by light microscopy.

The binding was performed as described in “Experimental Procedures” using untreated cultures or cultures treated with NGF (50 ng/ml) for 3 days.

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**Fig. 6.** NGF-induced down-regulation of EGF receptors and stimulation of Erk phosphorylation in stably transfected PC12nnr-p140

**Fig. 7.** The effect of K-252a on NGF-induced down-regulation of EGF receptors. A, PC12 cell cultures (2 × 105 cells/well) were treated with 0.01% dimethyl sulfoxide (●), 200 nM K-252a (○), 50 ng/ml NGF (▵), and 200 ng/ml NGF (○). B, 6.24 cell cultures (0.7 × 106 cells/well) were treated with 500 nM K-252a (○), 20 ng/ml NGF (▵), 5 ng/ml NGF (●), and 500 ng/ml K-252a plus 5 ng/ml NGF (○). The medium was changed every day, and K-252a was preincubated with the cultures for 1 h before NGF addition. The cultures were washed twice with fresh medium before the binding experiments were performed. The error bars indicate the S.D. NGF differs from control at each point in A and from 0 day in B with a p value of <0.05.

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NGF-induced Down-regulation of EGF Receptors in PC12 Cells

NGF-induced Down-regulation of EGF Receptors is Src-dependent—The SrcDN2 PC12 cell variant, overexpressing a kinase-inactive, dominant-negative Src was treated with NGF and 125I-EGF binding was measured (Fig. 9). These cells express 46% of the p140

NGF treatment for 4 days did not induce the down-regulation of EGF receptors (Fig. 9) typical of
FIG. 8. NGF- and EGF-stimulated down-regulation of EGF receptors and activation of Erk1 GSrasDN6 and M-M17-26 dominant-negative ras cells. A, specific binding of $^{125}$I-EGF to GSrasDN6 cells cultured in the absence (−DEX) or the presence (+DEX) of dexamethasone (1 μM) for 24 h followed by an additional 48 h of incubation with either 50 ng/ml NGF (2, 5), 20 ng/ml EGF (3, 6), or no further additions (4, B). $^{125}$I-EGF binding was performed as described under “Experimental Procedures” with M-M17-26 cell cultures untreated (open circles) or treated with 50 ng/ml NGF (filled circles) for 4 days. The error bars indicate the S.D. C, GSrasDN6 cells cultured in the absence (−DEX) or the presence (+DEX) of dexamethasone (1 μM) for 24 h, and M-M17-26 and PC12 cells were treated with 50 ng/ml NGF (N), 20 ng/ml EGF (E), or were left untreated (C). Erk1 was immunoprecipitated from clarified cell lysates (2.5 mg), and immune complex kinase assays using MBP as an exogenous substrate were performed as described under “Experimental Procedures.” The fold activity increase was calculated by densitometer and was as follows: GSrasDN (−DEX): N, 11.8; E, 11.5; (+DEX): N, 2.0; E, 1.5; M-M17-26 cells: N, 0.7; E, 0.6; PC12 cells: N, 5.2; E, 6.6. The autoradiograms shown are from a single representative experiment and are characteristic of results obtained from three independent experiments.

wild-type PC12 cells. As expected, Western blotting analysis also did not show any change in the level of EGF receptors in SsrcDN2 cells treated for 5 days with 50 ng/ml NGF compared with untreated cells. However, treatment of the cells with 50 ng/ml EGF for 1 h at 37 °C induced 86% homologous down-regulation of the EGF receptors (data not shown).

NGF-induced Down-regulation of EGF Receptors Is Independent of Neurite Outgrowth—In view of the observation that in wild-type PC12 cells, the NGF-induced down-regulation of EGF receptors is concomitant with NGF-induced neurite outgrowth, we cultured PC12 cells for 7 days either in monolayer or suspension cultures in the presence or absence of 50 ng/ml NGF. In monolayer cultures the cells grow neurites in the presence of NGF; in suspension they aggregate, but do not grow neurites (18). Thereafter the cells were harvested and analyzed by Western blotting with either anti-EGF receptor antibody to measure EGF receptor down-regulation or anti-neurofilament-M antibody to measure the efficiency of the NGF treatment of the cells. As seen in Fig. 10, NGF induced down-regulation of EGF receptors in suspension cultures in the absence of neurite outgrowth after 7 or 10 days of NGF treatment. Binding experiments with $^{125}$I-EGF indicated a decrease of 68 and 82% in EGF receptors in suspension cultures treated for 7 and 10 days with NGF, respectively, compared with 85% decrease in EGF receptors in monolayer cultures.

DISCUSSION

Both acute and chronic heterologous regulation of EGF receptors has been demonstrated in many cells. This regulation process is characterized by a change in either the affinity or the number of EGF receptors. For example, a decreased affinity of EGF receptors was measured upon treatment of cells with platelet-derived growth factor (23), vasopressin (24), interleukin 1 (25), and phorbol esters (26), and a decrease in the number of EGF receptors was observed in response to thyroid hormone (27), norepinephrine (28), nerve growth factor (11), or after infection with adenovirus (29, 30) or Rous sarcoma virus (31). In a few cases, the heterologous up-regulation of EGF receptors has been seen. An increased number of EGF receptors is evident in 3T3-L1 fibroblasts treated with 3-deazaadenosine (32), in teratocarcinoma cells treated with retinoic acid (33), or upon oncogenic transformation of carcinoma cells (34). The cellular signaling pathways utilized by G protein-coupled receptors, tyrosine kinase receptors, viruses, or drugs to regulate EGF receptors are as yet poorly characterized.

PC12 cells possess receptors for EGF, FGF, and NGF and have been used to investigate the process of heterologous down-regulation of EGF receptors by NGF. In these cells, both an acute (10 min) (35, 36), as well as a chronic (several days) (10, 11) down-regulation of EGF receptors in response to NGF has been reported. This biphasic down-regulation process was also
observed upon NGF-induced down-regulation of c-neu receptors by NGF in PC12 cells (16). The present data emphasize the three major characteristics of the NGF-induced, chronic down-regulation of EGF receptors in PC12 cells: (a) a decrease in receptor level on the cell surface; (b) a disappearance of EGF-stimulated, receptor-mediated tyrosine kinase activity; and (c) a decrease in receptor synthesis.

Mechanisms have been proposed for the acute NGF-induced down-regulation of EGF receptors (35, 36). Acute regulation of EGF receptors has been attributed to activation of either protein kinase C, which phosphorylates threonine 654 of the EGF receptor (37), or of calmodulin-dependent protein kinase II, which phosphorylates serine 1046/1047 (38), or of other unknown kinases (39, 40), which might be also the case for PC12 cells. On the other hand, the signaling pathway(s) utilized by NGF for the chronic down-regulation of EGF receptors has not been addressed. The chronic, NGF-induced down-regulation of EGF receptors in PC12 cells is definitely different in mechanism from the acute down-regulation. It is selectively induced by p140\(^{\text{Src}}\) receptors, but not by G protein-coupled PACAP receptors or drug (staurosporine)-induced neurite outgrowth. This process is not due to a change in receptor affinity (10, 11), receptor processing, or receptor internalization (11, 16). The lack of NGF-induced heterologous down-regulation of EGF receptors in PC12 cells is the partial reconstitution of the heterologous down-regulation in PC12 cells by p140\(^{\text{Src}}\) transfectants, and the ability of K-252a to block NGF-induced EGF receptor down-regulation support the concept that this effect is mainly mediated by the high affinity NGF receptor, p140\(^{\text{Src}}\), and not by the low affinity NGF receptor, p75\(^{\text{NGFR}}\). This conclusion is also supported by the ability of both Src and Ras dominant-negative forms to block this cellular process, since these two signal transduction pathways are initiated by binding of NGF to p140\(^{\text{Src}}\) receptors, but not by binding of NGF to p75\(^{\text{NGFR}}\) (5).

Src has been implicated, in PC12 cells, in calcium-stimulated signaling events (5, 6, 8) as well as in tyrosine phosphorylation of cytoskeletal proteins (41), either of which might also be involved in NGF-induced down-regulation of EGF receptors. Alternatively, since expression of the dominant-negative Src blocked the tyrosine phosphorylation of both Src homologous collagen protein and mitogen-activated protein kinases (5, 6), the inhibition of NGF-induced down-regulation of EGF receptors might simply reflect the blockade of the Ras signaling pathway. Because Fyn, Yes, and other tyrosine kinase members of the Src family could also be blocked by overexpression of the kinase-inactive Src (8), there is a possibility that these kinases, as well, may be involved in NGF-induced down-regulation of the EGF receptors.

One possible explanation of our findings is that Ras-erk activity is an obligatory element in p140\(^{\text{Src}}\)-mediated pathway required for chronic NGF-induced, down-regulation of EGF receptors, affecting an event such as phosphorylation of protein(s) involved in the transcription, translation, and/or post-translational modification of EGF receptors.

Alternatively, it is possible that Ras suppression of NGF-induced neurite outgrowth indirectly blocked NGF-induced down-regulation of EGF receptors. This possibility seems unlikely, however, because our data suggest a lack of correlation between NGF-induced down-regulation of EGF receptors and NGF-induced neurite outgrowth in different PC12 cell variants investigated: different agents, PACAP and staurosporine, induced neurite outgrowth but not down-regulation of EGF receptors, and in suspension where neurite outgrowth is not possible NGF treatment induces down-regulation of EGF receptors in the absence of neurite outgrowth.

Preliminary studies show a significant decrease in EGF receptor mRNA after treatment of cells with NGF for 5 days. Under comparable conditions, the stability of the mRNA is unchanged. These data indicate that at least part of the mechanism by which NGF down-regulates the EGF receptor is transcriptional, but further experiments are necessary to confirm and extend these observations.

In summary, our data are consistent with the signal transduction mechanism of p140\(^{\text{Src}}\) receptors (4) and strongly suggest a series of NGF-induced, neurite outgrowth-independent, p140\(^{\text{Src}}\)-Ras phosphorylation events in the down-regulation of EGF receptors in PC12 cells.

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REFERENCES

1. Guroff, G. (1985) Cell Culture in the Neurosciences, pp. 245–271, Plenum Publishing Corp., New York
2. Keegan, K., and Halegoua, S. A. (1976) Proc. Natl. Acad. Sci. U. S. A. 73, 2424–2428
3. Chao, M. V. (1992) Neuron 9, 583–593
4. Kaplan, L., and Rabin, S. J. (1994) J. Neurobiol. 25, 1404–1417
5. Keegan, K., and Hageoluova, S. (1993) Curr. Opin. Neurobiol. 3, 14–19
6. Kremer, S. E., D’Arcangelo, G., Morgan, S. R., and Guroff, G. (1993) J. Cell. Physiol. 155, 809–819
7. Szeberenyi, J., Cai, H., and Cooper, G. M. (1990) Mol. Cell. Biol. 10, 5324–5332
8. Rusanescu, G., Qi, H., Thomas, S. M., Brugge, J. S., and Hageoluova, S. (1995) J. Neurochem. 65, 1415–1425
9. Haff, K., End, D., and Guroff, G. (1981) J. Cell. Biol. 88, 189–198
10. Chandler, C. E., and Herschman, H. R. (1983) J. Cell. Physiol. 114, 321–327
11. Lazarovicz, P., Dickens, G., Kozuza, H., and Guroff, G. (1986) J. Cell. Biol. 104, 1611–1621
12. Hampstead, B., Rabin, S. J., Kaplan, L., Reid, S., Parada, L. F., and Kaplan, D. R. (1992) Neuron 9, 883–896
13. Green, S. H., Rydel, R. E., Connolly, J. L., and Greene, L. A. (1986) J. Cell. Biol. 102, 830–843
14. Thomas, S. M., deMarco, M., D’Arcangelo, G., Hageoluova, S., and Brugge, J. S. (1992) Cell 68, 1031–1040
15. Feig, L. A., and Cooper, G. M. (1988) Mol. Cell. Biol. 8, 3235–3243
16. Oshima, M., Shibutani, Y., Katagiri, and Guroff, G. (1995) J. Neurochem. 65, 427–433
17. Zimmer, Y., Givol, D., and Yan, A. (1995) J. Biol. Chem. 268, 7899–7903
18. Fujita, K., Guroff, G., Yavin, Y., Goping, G., Orinenberg, R., and Lazarovicz, P. (1990) Neurochem. Res. 15, 373–383
19. Lebien, T. W., Bess, D. R., Bradley, J. G., and Kersey, J. H. (1982) J. Immunol. 129, 2287–2292
20. Yakovenchko, E., Whalin, M., Movseyvan, Y., and Guroff, G. (1996) J. Neurochem. 67, 540–548
21. Leb, D. M., and Greene, L. A. (1993) J. Neurosci. 13, 2919–2929
22. Koizumi, S., Contreras, M. L., Matsuda, Y., Hama, T., Lazarovicz, P., and Guroff, G. (1995) J. Neurosci. 15, 715–721
23. Obrin, B., and Hauschka, S. D. (1988) J. Cell. Biol. 107, 761–769
24. Rozenburg, E., Brown, K. D., and Pettican, P. (1981) J. Biol. Chem. 256, 716–722
25. Bird, T. A., and Saklatvala, J. (1990) J. Biol. Chem. 265, 540–548
26. Shibutani, Y., Katagiri, and Guroff, unpublished data.

2. M. Shibutani, Y. Katagiri, and G. Guroff, unpublished data.
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30. Boulukos, K. E., and Ziff, E. B. (1993) Oncogene 8, 237–248
31. Wasilenko, W. J., Shawrer, L. R., and Weber, M. J. (1987) J. Cell Physiol. 131, 450–457
32. McCaffrey, P. G., Friedman, B., and Rosner, M. R. (1984) J. Biol. Chem. 259, 12502–12507
33. Rees, A. R., Adamson, E. D., and Graham, C. F. (1979) Nature 281, 309–311
34. Gullick, W. J., Marsden, J. J., Whittle, N., Ward, B., Bobrow, L., and Waterfield, M. D. (1986) Cancer Res. 46, 285–292
35. Brown, A. B., and Carpenter, G. (1991) J. Neurochem. 57, 1740–1749
36. Mothe, I., Ballotti, R., Tartare, S., Kowalski-Chauvel, A., and Van Obberghen, E. (1993) Mol. Biol. Cell 4, 737–746
37. Davis, R. J. (1988) J. Biol. Chem. 263, 9462–9469
38. Countaway, J. L., Nairn, A. C., and Davis, R. J. (1992) J. Biol. Chem. 267, 1129–1140
39. Countaway, J. L., Giriones, N., and Davis, R. J. (1989) J. Biol. Chem. 264, 13642–13647
40. Bird, T. A., and Saklatvala, J. (1989) J. Immunol. 142, 126–133
41. Khan, M. A., Okumura, N., Okada, M., Kobayashi, S., and Nakagawa, H. (1995) FEBS Lett. 31, 201–204