Transcriptional Down-regulation of Epidermal Growth Factor Receptors by Nerve Growth Factor Treatment of PC12 Cells*

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Treatment of PC12 cells with nerve growth factor leads to a decrease in the number of epidermal growth factor receptors on the cell membrane. The mRNA for the epidermal growth factor receptor decreases in a comparable fashion. This decrease appears due to a decrease in the transcription of the epidermal growth factor receptor gene because first, there is no difference in the stability of the epidermal growth factor receptor mRNA, second, newly transcribed epidermal growth factor receptor mRNA is decreased in nerve growth factor-differentiated cells, and third, constructs containing the promoter region of the epidermal growth factor receptor gene are transcribed much less readily in nerve growth factor-differentiated cells than in untreated cells. The decreases in mRNA are not seen in the p140<sup>trk</sup> deficient variant PC12nnr5 cells nor in cells containing either dominant-negative Ras or dominant-negative Src. Treatment with nerve growth factor also increases the cellular content of GCF2, a putative transcription factor inhibitory for the transcription of the epidermal growth factor receptor gene. The increase in GCF2, like the decrease in the epidermal growth factor receptor mRNA, is not seen in PC12nnr5 cells nor in cells expressing either dominant-negative Ras or dominant-negative Src. The results suggest that nerve growth factor-induced down-regulation of the epidermal growth factor receptor is under transcriptional control, is p140<sup>trk</sup>-Ras-, and Src-dependent, and may involve transcriptional repression by GCF2.

PC12, a cell line isolated from a rat pheochromocytoma (1, 2), has been an extremely informative tool for the study of the mechanism of nerve growth factor (NGF)<sup>1</sup> action. Under standard culture conditions, these cells are small round and fluorescent and divide about every 48 h. Upon the addition of NGF, they elaborate neurites, become electrically excitable, stop dividing, and will synapse with appropriate muscle cells in culture (3). Overall, the cells change from a chromaffin-like phenotype to one very similar to that of a mature sympathetic neuron. A great deal of attention has been directed toward the mechanism by which NGF instructs the cells to undergo this global change in character.

One interesting property of PC12 cells is the appearance of both NGF receptors and epidermal growth factor receptors (EGFR) on their surface (4). Because NGF inhibits PC12 cell division and epidermal growth factor (EGF) stimulates PC12 cell division (4), this observation has motivated a number of studies on comparative signal transduction in these cells. These experiments have led to the conclusion that the temporal aspects of cellular signaling, as well as the exact nature of the signaling components, are important in determining the eventual changes caused by a ligand on its target cell (5, 6).

Another question posed by this observation had to do with the consequences of treating the cells simultaneously with an agent that stops their growth and one that stimulates it (4). The result of such dual treatment was that the differentiating agent, NGF, caused a decrease in the receptors for the mitogen, EGF (4). Although the mechanism of this down-regulation was not known, it was suggested that the decrease was, at least in part, the way in which NGF instructed the cells to stop dividing and differentiate, by binding them to the mitogens that normally control their growth.

Recently it has been shown (7) that the down-regulation of the EGFR by NGF is dependent on the Ras-Raf-MAP kinase pathway. That is, the down-regulation does not occur in PC12 variants that cannot signal by this pathway. It was also shown that the down-regulation is mediated by the p140<sup>trk</sup> receptor. Finally, it was demonstrated that although the down-regulation accompanies NGF-induced morphological differentiation in these cells, it occurs whether or not the cells are allowed to differentiate morphologically. However, the molecular mechanisms controlling NGF-induced EGFR down-regulation remained unknown.

In the present work, we have shown that the down-regulation has a transcriptional basis. Further, we have demonstrated that this decreased transcription is mediated by the p140<sup>trk</sup> receptor and the Ras-Raf-MAP kinase pathway. Finally, we have found that increases in the transcription inhibitor GCF2 (transcriptional repressor of the epidermal growth factor receptor gene) accompany the decrease in the EGFR and that these increases are also mediated by p140<sup>trk</sup> and the Ras-Raf-MAP kinase pathway.

EXPERIMENTAL PROCEDURES

Materials—Mouse NGF and rat type I collagen were purchased from Becton Dickinson (Bedford, MA). Monoclonal antibody against the EGFR (6F1) was obtained from Medical and Biological Laboratories,
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Co., Ltd. (Nagoya, Japan). LipofectAMINE was purchased from Life Technologies, Inc. Actinomycin D was a product of Sigma). Stable dominant-negative Src PC12 variant cells (SrcDN2) were the kind gift of Dr. Simon Hagegoua, stable dominant-negative Ras PC12 variant cells (M-M17–26) were generously provided by Dr. Geoffrey Cooper, and PC12 cells (glioma growth factor) were generously contributed by Dr. Lloyd Greene.

A DNA fragment of human GCF2 (GenBank accession number U69690) corresponding to amino acids 51–705 that had been ligated with BamHI linkers was ligated into the BamHI site of pGEX1T (Pharmacia Biotech Inc.). The direction was confirmed by DNA sequencing. The human GCF2 glutathione S-transferase fusion protein (GST-GCF2) was purified on a glutathione-Sepharose affinity column from isopropylthiogalactoside-induced E. coli according to the manufacturer’s protocol. The polyclonal antibody was the protein A-Sepharose-purified IgG fraction from antisera raised in rabbits against GST-GCF2.

Cell Culture—PC12 cells were grown in Dulbecco’s modified Eagle’s medium supplemented with 5% fetal bovine serum, 10% horse serum. For NGF treatment, 100 ng of NGF/ml were added to the culture medium. In all experiments involving extended treatment with NGF, the medium was changed and fresh NGF was added every other day. PC12nnr5 cells (8) were grown in RPMI 1640 medium (Life Technologies, Inc.) supplemented with 5% fetal bovine serum and 10% horse serum. The PC12 cell variants M-M17–26 expressing the Hα receptor were grown in RPMI 1640 medium supplemented with 5% fetal bovine serum, 10% horse serum, 100 ng of NGF/ml were added to the culture medium. In all experiments involving extended treatment with NGF, the medium was changed and fresh NGF was added every other day. PC12nnr5 cells (8) were grown in RPMI 1640 medium (Life Technologies, Inc.) supplemented with 5% fetal bovine serum and 10% horse serum.

Total cytoplasmic RNA was isolated from untreated and NGF-treated PC12 cells using FastTrack (In-vitrogen, San Diego, CA). The RNA was analyzed on 0.8% formaldehyde-agarose gels, transferred to Hy Bond-N nylon membranes, and probed with a 32P-random primed-0.8 kb PstI fragment of the rat EGFR (nucleotides 463–1311; the kind gift of Dr. H. Shelton Earp), followed by autoradiography. After stripping with a boiled 0.1% SDS solution, the membrane was reprobed with a 0.68-kb fragment of rat GAPDH (nucleotides 379–1060).

Competitive RT-PCR—Total cytoplasmic RNA was isolated from untreated control PC12 cells and NGF-treated PC12 cells by RNA STAT-60 (Tel-Test “B”, Inc., Friendswood, TX). Single strand DNA was generated from 1 μg of total RNA with Superscriptase Preamplification System (Life Technologies, Inc.). PCR MIMIC for competitive RT-PCR was made utilizing a construction kit according to the manufacturer’s protocol (CLONTECH, Palo Alto, CA). The target fragment (nucleotides 154–843) derived from EGFR was amplified using an upstream primer, 5′-TGGAGGGCGT GGCACGGCGCAGACACG-3′, an downstream primer, 5′-TGGCTCTAAGG GACTGGCGCAC GAAAC-3′, the size of the PCR products the EGFR target and the PCR MIMIC were 690 and 538 bp, respectively. The GAPDH sequence (nucleotides 379–1060) was amplified using an upstream primer, 5′-TGGAGGGCGT GGGCGCAGACACG-3′, and a downstream primer, 5′-GCTGATCGACCG ACTGACGAC-3′, and PCR products of the GAPDH target and the PCR MIMIC were 682 and 488 bp, respectively. Six tubes of 2-fold serial dilutions of PCR MIMIC were prepared for each competitive PCR sample. The cycle parameters for PCR were 94 °C for 1 min, 56 °C for 1 min, and 72 °C for 1 min, and the cycle numbers for EGFR and GAPDH were 24 and 20, respectively.

Nuclear Run-on Transcription—The double stranded cDNA clone for rat NGFI-B, and rat GAPDH were used as templates for hybridization. The 1.35-kb Nsi-I-PstI fragment of the rat EGFR (nucleotides 462–1815) and the 1.46-kb Nart-Nhel fragment of rat NGFI-B were each ligated into pBluescript II KS(+) utilizing its PstI site and ClaI/XhoI sites, respectively. The GAPDH fragment, which is identical to the target fragment used for competitive RT-PCR, was also ligated into pBluescript II KS(+) 2 μg of linearized template DNA was immobilized on GeneScreen nylon membrane (NEN Life Science Products) according to the manufacturer’s instructions, using a slot blot apparatus (Schleich & Schuell).

Nuclei were resuspended from untreated PC12 cells or PC12 cells treated with NGF (100 ng/ml) or with actinomycin D (10 μM) were prepared using Nonidet P-40 lysis buffer (10 mM Tris-HCl, pH 7.4, 10 mM NaCl, 3 mM MgCl2, 0.5% (v/v) Nonidet P-40) according to standard methodology (11). Isolated nuclei were resuspended in 10 mM EDTA, 10 mM sodium phosphate, pH 6.8, 5% glycerol, 5 μg/ml RNAse A, 0.1 mM EDTA, and samples of 2 × 107 nuclei in 40 μl were flash-frozen in ethanol and dry ice and stored at –70 °C. Nuclear run-on transcription assays were carried out at 24 °C for 20 min by the addition of 70 μl of 2× reaction buffer (10 mM Tris-HCl, pH 8.0, 5 mM MgCl2, 0.3 mM KCl, 1 mM ATP, 1 mM GTP, 1 mM CTP), 27 μl of [α-32P]UTP (50 μCi, 3000 Ci/mmol, Amersham Corp.), and 3 μl of labeled template DNA. The reaction was stopped by DNA digestion at 24 °C with 20 μl of RQ1 RNase-free DNase (20 units, Promega) and 18 μl of 10 mM CaCl2 for 5 min, followed by digestion with proteinase K (1 μl containing 14.4 μg, Boehringer Mannheim) at 42 °C for 30 min, and then the addition of 20 μl of 10× SDS/Tris buffer (100 mM Tris-HCl, pH 7.4, 5% SDS, 50 mM EDTA) and 10 μl containing 100 μg of yeast tRNA at 37 °C for 15 min. The labeled nascent RNAs were isolated by RNA STAT-60 and ProbeQuant™ G-50 Micro Columns (Pharmacia) and were partially hydrolyzed by incubation with 0.1 % NaOH on ice for 10 min. The mixture was neutralized with Tris-HCl and precipitated with ethanol. The labeled RNA was resuspended in 100 μl of 10 mM Tris-HCl, pH 7.4, 1 mM EDTA, and 0.1% SDS and denatured at 95 °C for 10 min before hybridization. Labeled RNA (6 × 106 cpm) was hybridized to the immobilized template DNAs at 52 °C in hybridization solution (6% SSPE, 0.1% SDS, 0.1% BSA, 0.1% SDS, 0.1% BSA, 0.1% SDS) at 55 °C for 20 hr. The filters were washed for 20 min once in 2× SSC, 0.1% SDS at 60 °C, twice in 2× SSC, twice in 1× SSC containing RNase A 1 μg/ml at room temperature, and twice in 1× SSC containing 0.1% SDS at 60 °C and 0.2× SSC containing 1.0% SDS at 60 °C. Filters were processed for PhosphorImager analysis using STORM860 (Molecular Dynamics) and analyzed by autoradiography.

Reporter Gene Assay—The plasmids pER6-luc, pER9-luc, and pER10-luc (12) were obtained by inserting the promoter regions from pERCAT6, pERCAT9, and pERCAT10 into the HindIII site of the pEL-basic plasmid (Promega). PC12 cells cultured in collagen-coated 6-well plates (Nunc, Naperville, IL) were transfected using LipofectAMINE with 1 μg of pER-luc plasmid and 0.1 μg of the internal control pRL-TK (Promega), which contains Renilla luciferase downstream of the Herpes simplex virus-thymidine kinase promoter. After NGF treatment (100 ng/ml) for various periods of time, cells were transfected for 1 h. 9 hr after transfection, cell lysates were prepared and luciferase activity was measured using a luciferase assay system (Promega), and both firefly and Renilla luciferase activity were measured in an LB 9507 luminometer (Berthold, Wildbad, Germany). The transfection efficiency was normalized by the Renilla luciferase activity. The data are expressed as the means ± S.D. In some experiments, RNA template specific PCR of the luciferase gene from PC12 cells transfected with pER6-luc was used (13). Total cytoplasmic RNA from transfected cells was isolated with an acidic phenol/chloroform extraction and the luciferase region is complementary to a region of the target luc gene (nucleotides 1614–1593 of pGL3-Basic) and its 3′-region is complementary to a region of the luc gene (nucleotides 1614–1593 of pGL3-Basic) and its 5′-region is a tagged sequence. PCR was performed with the sequence-specific first strand cDNA as a template, an upstream primer, 5′-ACCTGCAGCT TCTGGT-GGGC TTCGCCACCTC-3′, and a downstream primer, 5′-GGGCGGCGCC TCTGAAACTA GTGCGG-3′, where its 3′-region is complementary to a region of the target luc gene (nucleotides 1614–1593 of pGL3-Basic) and its 5′-region is a tagged sequence. PCR was performed with the sequence-specific first strand cDNA as a template, an upstream primer, 5′-ACCTGCAGCT TCTGGT-GGGC TTCGCCACCTC-3′, and a downstream primer, 5′-GGGCGGCGCC TCTGAAACTA GTGCGG-3′.
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RESULTS

NGF-induced Down-regulation of EGFR—The level of EGFR during NGF-induced differentiation of PC12 cells was examined by Western blot analysis (Fig. 1). A reduction in the level of the EGFR protein in whole cells became apparent after 1 day of NGF treatment, and this reduction was gradual and progressive. A 67% decrease was seen after 5 days of treatment. This reduction was consistent with the reduction in [125I]EGF binding during NGF-induced differentiation observed in previous reports from this laboratory (7).

NGF-induced Down-regulation of EGFR mRNA—Fig. 2 shows the levels of EGFR mRNA during NGF-induced differentiation determined both by Northern blot analysis (Fig. 2A) and by competitive RT-PCR (Fig. 2, B and C). The time points are the same as those in Fig. 1. Both methods show a progressive reduction in EGFR mRNA levels during NGF treatment. Three species, one major (9.6 kb) and two minor (6.5 and 5.0 kb), of EGFR mRNA have been reported (14), along with a 2.7-kb species considered to be a truncated variant, the protein product of which lacks both transmembrane and intracellular domains. Northern blot data show that all three species decrease in the course of NGF treatment, and this decrease precedes the reduction in the protein. Competitive RT-PCR, a quantitative procedure for analyzing mRNA levels that is also more sensitive than RNA blot techniques, such as Northern blotting, which provide only semi-quantitative results (15), shows a very similar decrease in EGFR mRNA. Fig. 2B depicts the actual PCR products visualized on gels obtained from untreated control cells and from cells treated for 5 days with NGF.

The arrows in Fig. 2B indicate the cross-over points as determined visually. Even by visual estimation, a shift in the cross-over point could be clearly seen. The relative level of EGFR...
mRNA at each time point was estimated by the quantitation of ethidium bromide-stained gel bands (Fig. 2C). The region selected as a target fragment for PCR amplification was almost identical to the sequence of the EGFR DNA fragment used as a probe for Northern blot analysis. The decrease in EGFR mRNA measured by competitive RT-PCR began after 6 h of NGF treatment (Fig. 2C), and an 85% reduction was seen after 5 days of NGF treatment, a result very similar to that obtained by Northern blot analysis. The reduction became statistically significant after 1 day of treatment. GAPDH mRNA, the internal control, was constant during NGF treatment.

**EGFR mRNA Stability**—The decay of EGFR mRNA in untreated control cells and in cells treated for 5 days with NGF was measured in the presence of actinomycin D by competitive RT-PCR (Fig. 3). The decay of EGFR mRNA was faster than that of GAPDH mRNA. Neither EGFR mRNA nor GAPDH mRNA showed any significant changes in their decay due to NGF treatment. The same result was obtained by Northern blot analysis of samples from cells treated for 3 or 5 days with NGF (data not shown).

**Nuclear Run-off Transcription of EGFR Gene**—The nascent transcript levels of EGFR were measured in untreated control cells and in cells treated with NGF for 45 min or 5 days (Fig. 4). Two different experiments were performed, and the data obtained from these two experiments were almost identical. The transcription of EGFR was decreased by 55% in PC12 cells treated with NGF for 5 days. By way of control, a 2-fold increase in NGFI-B transcription was observed after 45 min of NGF treatment. NGF treatment had no effect on the transcription of the housekeeping gene, GAPDH, and there were no transcripts from any of these genes in cells treated with actinomycin D.

**NGF-induced Decrease in EGFR Promoter Activity**—To study the EGFR promoter activity and to confirm the decrease in the transcriptional activity in NGF-treated cells, three luciferase gene plasmids with different lengths of promoter were used for reporter gene assay of PC12 cells. Compared with the promoter activities from the longer promoter plasmids (pER6-luc and pER9-luc), the activity of the shortest promoter plasmid (pER10-luc) was very low (Fig. 5A). After NGF treatment, the normalized EGFR promoter activity of pER6-luc and pER9-luc transfectants was almost completely repressed (Fig. 5B). The decrease in the promoter activity of pER10-luc was not as strongly inhibited. To evaluate the time course of the reduction in the promoter activity, PC12 cells were treated with NGF for different periods of time before transfection. Even 12 h after NGF treatment, a significant reduction in promoter activity was observed, and this activity decreased with time (Fig. 5C).

The slow but progressive decrease in the promoter activity was consistent with the changes in EGFR mRNA seen during NGF treatment (Fig. 2). Table I shows the effect of introducing different amounts of promoter plasmid DNA into the cells on the extent of NGF-induced down-regulation of EGFR promoter activity. Untreated PC12 cells and cells treated with NGF (100 ng/ml) for 5 days were transfected with either 1 µg or 0.1 µg of pER9-luc EGFR promoter DNA, and luciferase assays were performed. The down-regulation of EGFR promoter activity was seen for up to 15 h after transfection with 1 µg of DNA but disappeared after 24 h. On the other hand, transfection with 0.1 µg of DNA showed the down-regulation even 24 h after the transfection.

**Transcriptional Control of NGF-induced Decrease in pER6-luc Expression**—Because the NGF-induced decrease in luciferase activity could occur at either the transcriptional or the translational level, the levels of luciferase mRNA were measured by RNA template-specific PCR. Fig. 6A shows an experiment validating the PCR measurement. With the combination of the upstream and tagging primers, sequences derived from luciferase mRNA that had been tagged with unique sequence during reverse transcription are amplified preferentially, whereas contaminating DNA derived from the transfected plasmid that lacks the unique tag is not amplified. Lane 1 of Fig. 6A shows the result of PCR reaction of pGL3-Basic as a template with the combination of the upstream primer and the tagging primer. Lane 2 of Fig. 6A shows the product (597 bp) amplified by the PCR reaction of pGL3-Basic as a template with the combination of the upstream primer and the downstream chimeric primer. Lane 3 of Fig. 6A shows the product (622 bp) amplified by the reaction of single strand DNA derived from pER6 transfectant as a template with the combination of the upstream primer and tagging primer. From these results, PCR with the combination of upstream primer and tagging primer selectively amplifies luciferase mRNA. Fig. 6B shows the RNA template-specific PCR products from the pER6-luc transfected PC12 cells after 5 days of culture in the presence or the absence of NGF. Compared with untreated control cells (Fig. 6B, lane 1), NGF-treated cells showed much lower amplification of the PCR product (Fig. 6B, lane 2), demonstrating that the regulation of the expression of this plasmid is at the transcriptional level.

**Involvement of p140trk in Transcriptional Repression of EGFR**—PC12nnr5 cells are a variant of PC12 cells that express...
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Effect of the amount of transfected promoter DNA on the NGF-induced down-regulation of EGFR promoter activity

Untreated PC12 cells or cells treated with NGF for 5 days were transfected with either 1 μg or 0.1 μg of pER9-luc plasmid DNA and 0.1 μg of internal control pRL-TK. Cells were harvested after different periods of time, and luciferase assays were performed. Promoter activities are presented as percentages of untreated control and are the means ± S.D. of triplicate values. The 0.1 μg of promoter DNA differs significantly from the 1 μg of promoter DNA at 24 h.

| Time after transfection | 1 μg of promoter DNA | 0.1 μg of promoter DNA |
|-------------------------|----------------------|------------------------|
| h                       |                      |                        |
| 9                       | 13.8 ± 2.7           | 23.0 ± 13.6            |
| 15                      | 43.7 ± 5.9           | 26.0 ± 13.5            |
| 24                      | 108.0 ± 11.5         | 47.7 ± 14.8            |

* p < 0.05.

Fig. 5. EGFR promoter activity in untreated control PC12 cells and in cells treated with NGF. A, relative promoter activity of the human EGFR gene and of deletion mutants in PC12 cells. Chimeric reporter genes with various lengths of the EGFR promoter (pER6-luc, pER9-luc, and pER10-luc) were generated by inserting the promoter region from pERCAT6, pERCAT9, and pERCAT10 into the pGL3-Basic vector at the HindIII site. 1 μg of each reporter gene plasmid and 0.1 μg of the internal control pRL-TK were transfected into PC12 cells. 1 h after transfection, the solution was removed, and culture medium was added. 9 h after transfection, the cells were harvested, and luciferase activity was measured. Relative promoter activity measured by firefly luciferase activity was normalized by Renilla luciferase activity derived from pRL-TK. The values are the means of triplicate values. Luciferase activity of the empty vector (pGL3-Basic) was 7% of the activity obtained from the pER6-luc transfection. B, EGFR promoter activity in NGF-treated PC12 cells. After 5 days in culture in the presence or absence of NGF, PC12 cells were transfected with 1 μg of either pER6-luc, pER9-luc, or pER10-luc and 0.1 μg of pRL-TK. After transfection the solution was removed, and culture medium was added. 9 h after transfection the cells were harvested, and luciferase activity was measured. NGF was present throughout for the NGF-treated cells. Cells were untreated or were treated with 100 ng/ml of NGF for 5 days, and total RNA was prepared from cells 5 h after the transfection. 1 μg of total RNA was used for reverse transcription with the chimeric primer. After the reverse transcription reaction, PCR was performed using a combination of the upstream primer and the tagging primer. Lane 1, untreated control PC12 cells; lane 2, NGF-treated PC12 cells.

Table I

| Time after transfection | Relative promoter activity |
|-------------------------|---------------------------|
|                        | 1 μg of promoter DNA      | 0.1 μg of promoter DNA  |
| h                       |                           |                         |
| 9                       | 13.8 ± 2.7                | 23.0 ± 13.6             |
| 15                      | 43.7 ± 5.9                | 26.0 ± 13.5             |
| 24                      | 108.0 ± 11.5              | 47.7 ± 14.8             |

* p < 0.05.

Fig. 6. NGF-induced changes in the luciferase reporter gene mRNA levels in PC12 cells transfected with pER6-luc. A, validation of PCR primers used to amplify the luciferase RNA template derived from pER6-luc transfected cells. Reverse transcription was performed with the chimeric primer composed of the luciferase gene-specific and the tagged sequences. The upstream primer was designed utilizing the 5′-luciferase gene-specific sequence. With the combination of the upstream and tagging primers, sequences derived from luciferase mRNA that had been tagged with unique sequence during reverse transcription are amplified preferentially, whereas contaminating DNA derived from transfected plasmid that lacks the unique tag is not amplified. Lane 1 shows the result of PCR reaction of pGL3-Basic as a template with the combination of the upstream primer and the tagging primer. Lane 2 shows the product (597 bp) amplified by the PCR reaction of pGL3-Basic as a template with the combination of the upstream primer and the downstream chimeric primer. Lane 3 shows the product (622 bp) amplified by the reaction of first strand cDNA derived from pER6 transfectant as a template with the combination of the upstream primer and tagging primer. B, RT-PCR of pER6-luc transfected PC12 cells. Cells were untreated or were treated with 100 ng/ml of NGF for 5 days, and total RNA was prepared from cells 5 h after the transfection. 1 μg of total RNA was used for reverse transcription with the chimeric primer. After the reverse transcription reaction, PCR was performed using a combination of the upstream primer and the tagging primer. Lane 1, untreated control PC12 cells; lane 2, NGF-treated PC12 cells.

mRNA (Fig. 7B). PC12mr5 cells also did not show a significant reduction in EGFR promoter activity after 5-day treatment with NGF (Fig. 7C).

NGF-induced Increase in GCF2 Levels—GCF2 is a recently identified transcriptional repressor of the EGFR gene, which migrates as a 160-kDa protein on polyacrylamide gels. To examine the expression level of this protein in PC12 cells during NGF treatment, Western blot analysis was performed. As shown in Fig. 8, GST fusion protein expressed in a bacterial system was recognized by an antibody raised against GCF2. In PC12 cells, a 160-kDa major protein band and a minor protein band at about 150 kDa were detected. The level of these proteins was clearly increased by NGF treatment in a time-dependent manner.
PC12 cells are dependent upon both Ras and Src (7). To begin to explore the relationship between the increase in GCF2 and the decrease in the EGFR, the NGF-induced increase in GCF2 was inspected for its dependency on Ras or Src. Two PC12 cell variants, one stably overexpressing the dominant-negative mutant Ras\textsuperscript{N17} (M-M17–26), the other stably overexpressing a kinase-inactive, dominant-negative Src (SrcDN2) were treated with NGF for 5 days, and Western blot analyses of GCF2 and EGFR were performed. Fig. 9 shows the data from these variants and from PC12nnr5 cells as well. Unlike in the wild-type, there was neither an up-regulation of GCF2 levels nor a decrease in EGFR levels in these variant cell lines upon NGF treatment. It is interesting to note the very high basal levels of GCF2 in the Src dominant-negative cells and the absence of the 160-kDa GCF2 band in PC12nnr5 cells, even after NGF treatment.

**DISCUSSION**

The observation that both mitogenic and anti-mitogenic receptors occur on the same cell and the further finding that the presence of the ligand for the anti-mitogenic receptor, NGF, cause a profound decrease in the levels of the receptor for the mitogen, EGF, permitted the suggestion that this down-regulation could be one way in which NGF instructs its target cells to stop dividing and differentiate (4). The mechanism by which this down-regulation occurs has not been described. There have been two studies (17, 18) dealing with rather short term NGF-induced changes in EGFR levels on PC12 cells, but these clearly deal with a different phenomenon than the one described here.

The evidence that this regulation is exerted at the transcriptional level seems quite persuasive. There is a decrease in EGFR mRNA levels comparable with the decrease seen in the receptor itself. This decrease is clearly not caused by any difference in mRNA stability. Direct proof was obtained by nuclear run-off assay in which about 50% reduction of the nascent mRNA for the EGFR was observed as a result of long term treatment of the cells with NGF. Furthermore, evidence to support the transcriptional regulation was provided by transfecting EGFR promoter constructs linked to luciferase into untreated control and NGF-treated cells. The decreased luciferase activity in the treated cells, together with the direct measurement of luciferase mRNA to confirm that the decrease was at the transcriptional level, provides conclusive proof of the transcriptional regulation of this expression.

It should be noted that this decrease in expression was evident only for short times after the transfection. By 24 h after transfection there was no difference between the NGF-treated cells and the controls. That this was not due to differences in transfection speed or efficiency was clear from the clear run-off assay in which about 55% reduction of the nascent mRNA was observed at every time point in untreated control and NGF-treated cells.

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It should be noted that this decrease in expression was evident only for short times after the transfection. By 24 h after transfection there was no difference between the NGF-treated cells and the controls. That this was not due to differences in transfection speed or efficiency was clear from the Renilla controls, which were expressed equally at every time point in untreated control and NGF-treated cells. A possible explanation for these data is that the large amounts of EGFR promoter
region that are produced in the cells simply exhaust the inhibitory transcription factors available; such depletion has been suggested by observations with the transfected chromogranin A promoter in PC12 cells (19), in which the high dose of the transfected promoter DNA may saturate and deplete the transacting factor. Data consistent with such a possibility are presented in Table I in which transfection with a low dose of EGFR promoter DNA resulted in down-regulation even 24 h after transfection.

The regulation of EGFR expression is complex, involving at least five stimulatory transcription factors, Sp1 (21, 22), TCF (23), RPF-1 (24), and p53 (25, 26), and four inhibitory transcription factors, GCF1 (27), GCF2,2 ETR (28), and WT1 (29). Interactions of NGF with Sp1 and with p53 have been reported before. NGF has been shown to induce a subunit of the N-methyl-D-aspartate receptor (30) and the gene for the light neurofilament protein (31), at least in part, through an Sp1 site and the apoptotic death of PC12 cells after NGF withdrawal appears to lower Sp1 levels (32). NGF also appears to interact with p53 in that PC12 cells overexpressing p140<sup>Raf</sup> show an association between the receptor and p53, and p53 can induce an NGF-like response in these cells in the absence of NGF (33). Further, 3T3 cells show a Raf-dependent phosphorylation of p53 and a potentiation of the transactivation potential of p53 (16), and Raf is part of the Ras-Raf-MAP kinase pathway activated by NGF. But this is the first report of an NGF-induced alteration in one of the inhibitory factors.

GCF2 was identified by differential hybridization and library screening<sup>2</sup> using a cDNA for GCF1 (27). Contransfection assays show that GCF2 acts to repress transcription from the EGFR promoter as well as from those for SV40 and Rous sarcoma virus. Gel shift assays using His-tagged GCF2 protein have been presented (28), and Raf is part of the Ras-Raf-MAP kinase pathway activated by NGF. But this is the first report of an NGF-induced alteration in one of the inhibitory factors.

GCF2 was identified by differential hybridization and library screening<sup>2</sup> using a cDNA for GCF1 (27). Contransfection assays show that GCF2 acts to repress transcription from the EGFR promoter as well as from those for SV40 and Rous sarcoma virus. Gel shift assays using His-tagged GCF2 protein have revealed two binding sites in the human EGFR promoter: one is a strong binding site (−384 to −164 relative to the AUG translation initiation codon) and the other is a weaker binding site (−154 to −15).<sup>2</sup> Both pER6-luc and pER9-luc constructs have the promoter region that includes both binding sites, but pER10-luc contains only the weak binding site. These observations could explain the much weaker effect of NGF treatment on the transcription of pER10-luc. Clearly, the data presented here do not prove that GCF2 is involved in the decreased expression of the EGFR in NGF-treated PC12 cells, but the coincidence of the increased expression of GCF2 with the decreased expression of the EGFR and the fact that both events are dependent on p140<sup>Raf</sup>, Ras, and Src are consistent with that possibility. Further experiments testing that possibility are underway.

In any case, the decreased expression of the EGFR in PC12 cells caused by NGF differentiation is clearly transcriptional in nature. The increasing number of factors that appear to control that transcription indicate that the regulation is quite complex. But because the expression of EGFR and its homologs, the ErbB family, appear to be involved in the control of the growth of a number of tumors, the details of the control of that expression would seem to be worth pursuing.

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