Activation of the *neu* Tyrosine Kinase Induces the *fos/jun* Transcription Factor Complex, the Glucose Transporter, and Ornithine Decarboxylase

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Abstract. We have studied the ability of the *neu* tyrosine kinase to induce a signal for the activation of cell growth-regulated genes. Serum-starved NIH 3T3 cells expressing an epidermal growth factor receptor (EGF-R)/*neu* construct encoding a hybrid receptor protein were stimulated with EGF and the activation of the *neu* tyrosine kinase and stimulation of growth factor inducible genes were followed at the mRNA, protein, and activity levels, and compared to the corresponding responses in the *neu* proto-oncogene and oncogene expressing cells. Induction of the expression of *jun* mRNAs was an immediate early effect of EGF stimulation, followed by a marked increase in the biosynthesis of the *fos/jun* transcription factor complex and an increased transcription factor activity as measured by a recombinant transcription unit using chloramphenicol acetyltransferase assays. In distinction, elevated AP-1/PEA-1 activity in the absence of a significant increase in *jun* and *fos* expression was characteristic of the *neu* oncogene-expressing cells. The glucose transporter mRNA increased at 2 h of EGF stimulation and was associated with enhanced glucose transport of the EGF-treated cells. An increase of ornithine decarboxylase (ODC) mRNA and activity followed these changes. In contrast, serum-starved, EGF-treated *neu* proto-oncogene- and oncogene-expressing cells showed constitutively low and high glucose transporter and ODC activities, respectively. These findings demonstrate that the chimeric EGF-R/*neu* receptor is capable of activating the expression of both immediate early genes and biochemical activities associated with cell growth stimulation.

QUIESCENT cells are rendered capable of traversing the cell cycle by a cascade of events initiated by the binding of growth factors to their receptors at the cell surface and resulting in the activation of genes and biochemical activities expressed during the G_1 and S phases of the cell cycle (Heldin and Westermark, 1984; Lau and Nathans, 1987; Almendral et al., 1988). However, growth factors differ in their effects on different cells. They can be classified into at least two categories, called the competence and progression factors, depending on whether they are sufficient for the transfer of stimulated cells from a quiescent state (G_0) back to the cell cycle or whether they are required later, during cell progression into the S phase (Harrington and Pledger, 1987). This specificity of growth factor action is determined by the specific growth factor receptors, their effector functions, signal transduction mechanisms, and substrates, which are reflected at the genomic level in the activation of cell- and growth factor-specific gene responses. Oncogenes can deregulate signal transduction mechanisms and growth factor inducible genes at various levels and prevent cells from entering a quiescent stage.

The biochemical mechanisms of signal transduction used by many growth factor receptors remain uncharacterized in the absence of known ligands for several growth factor receptor tyrosine kinases encoded by genes that have been cloned recently. Thus, the protein product of the *neu* oncogene has been assumed to function as a tyrosine kinase growth factor receptor because of its close homology and immunochemical similarity with the epidermal growth factor receptor (EGF-R)[1] (Schechter et al., 1984; Bargmann et al., 1986a). Consistent with this idea, the rat *neu* protein, activated by a point mutation in the transmembrane domain of the putative receptor, has a tyrosine kinase activity (Bargmann et al., 1986b) as does its human homologue, the HER2/*erb*B2 protein, when overexpressed in NIH 3T3 cells (Hudziak et al., 1987). It is not known, however, how the function of the *neu* oncoprotein differs from its normal counterpart and what is the mechanism of cell transformation by the mutant protein.

We have undertaken a study of the function of the *neu* tyrosine kinase by expressing a chimeric EGF-R/*neu* protein in NIH 3T3 cells and analyzing its tyrosine kinase activity and effects in cells stimulated by EGF (Lehväslaiho et al., 1989). This study has indicated that the ligand-activated *neu* tyrosine kinase is capable of inducing DNA synthesis in resting

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1. *Abbreviations used in this paper*: CAT, chloramphenicol acetyltransferase; CHX, cycloheximide; 2-DOG, 2-deoxyglucose; EGF, epidermal growth factor; EGF-R, EGF receptor; GT, glucose transporter; ODC, ornithine decarboxylase; TRE, 12-O-tetradecanoyl-phorbol-13-acetate-responsive element.

cells and causes their transformation in the presence of receptor overexpression (Lehtola et al., 1989; Lehväslaiho et al., 1989). We have now initiated a more detailed analysis of the effects of the ligand-activated *neu* tyrosine kinase on several growth-regulated genes and biochemical functions. Using our chimeric receptor system, we could also compare the status of expression of these genes and activities in the *neu* proto-oncogene- and oncogene-expressing cells.

Materials and Methods

Cells and Transfections

The NIH 3T3 cells expressing less than 8×10^3 EGF receptors/cell were transfected with the pSV2EGF-R/*neu* vector (Lehväslaiho et al., 1989) according to the calcium phosphate precipitation technique described by Graham and van der Eb (1973). The following cell clones expressing various levels of the chimeric EGF-R/*neu* protein were obtained: NEN7 (2×10^6 receptors/cell), NEN16 (4×10^5 receptors/cell), NEN37 (4×10^5 receptors/cell), and NEN49 (5×10^5 receptors/cell). The NN cells expressing only the neomycin resistance marker gene were used as control cells in most experiments, since all transfections included the marker plasmid pSV2neo (No. 37149; American Type Culture Collection, Rockville, MD). We also produced various *neu*-transfected cell clones for control experiments: the N6 cells express the *neu* proto-oncogene (LTR*neu*N) while the NT8, NT11 and NT12 cells express the *neu* oncogene differing from the proto-oncogene by a single point mutation in the transmembrane domain (SV2*neu*NT; Bargmann et al., 1986b). The C125 cells are NIH 3T3 derivatives expressing ∼4 $\times 10^5$ human EGF-R/cell (Velu et al., 1987).

The cells were grown at 37°C in DME supplemented with 10% FCS. For selection of the transfectants, the G418 antibiotic was added at a concentration of 200–400 μg/ml. Before each growth factor stimulation experiment, the cells were washed with serum-free medium and incubated in 0.5% FCS for 16–48 h. The cells were routinely checked for mycoplasma contamination using fluorochrome 33 258 (Hoechst; Calbiochem-Behring Corp., San Diego, CA) (Russell et al., 1975), with negative results.

Reagents

Receptor-grade EGF from mouse submaxillary glands was obtained from Collaborative Research, Inc. (Waltham, MA) ^{125}I-labeled mouse EGF (100 μCi/μg), γ[32P]ATP (>5,000 Ci/mmol), [35S]methionine (1,000 Ci/mmol), 2-deoxy-D[1-3H]glucose (17 Ci/mmol), L-[1-14C]ornithine (59 mCi/mmol), and D-threo-[dichloroacetyl-1-14C]chloramphenicol (54 mCi/mmol) were from Amersham Corp. (Arlington Heights, IL); protein A-Sepharose, acetyl coenzyme A, and chloramphenicol acetyltransferase (*Escherichia coli*) (Pharmacia Fine Chemicals, Uppsala, Sweden), D luciferin from Enzymatix Ltd. (Cambridge, UK), and cell culture reagents including the neomycin analogue geneticin (G418 sulphate) from Gibco Laboratories (Grand Island, NY).

Molecular Clones

The proto-oncogene *neu* cDNA and SV40 polyadenylation site from plasmid pSV2neuN (Bargmann et al., 1986b) and Moloney murine leukemia virus LTR from plasmid pMP-1 (Schwab et al., 1985) were joined together in pSP72 (Promega Biotec, Madison, WI)) cloning vector to yield the expression vector pLTR*neu*N.

The following cDNA clones were used as molecular probes: pODC 16 (mouse ornithine decarboxylase; Jänne et al., 1984), pGT4-12 (rat glucose transporter; Birnbaum et al., 1986). p465.20 (*jun*B; Lau and Nathans, 1987), ph-cJ-1 (*c-jun*; Angel et al., 1988a), pRGAPDH-13 (rat glyceraldehyde phosphate dehydrogenase; Fort et al., 1985). Nick translations of DNA were carried out according to the manufacturer's instructions (Amersham Corp.). Plasmid pBLCAT2 containing the herpes virus thymidine kinase promoter in front of the chloramphenicol acetyltransferase coding sequence (CAT) and plasmid p5xTREtkCAT where a synthetic, fivefold human metallothionein IIA TPA-responsive DNA element (TRE: GTG-ACTCAG) is inserted in front of the thymidine kinase promoter in pBLCAT2 were kind gifts from Dr. Peter Angel (Angel et al., 1987). Plasmid pRSVL contains the full-length, intronless luciferase gene isolated from the firefly *Photinus pyralis*, placed under control of the Rous Sarcoma virus LTR (de Wet et al., 1987). This plasmid was kindly provided by Dr. Suresh Su-

bramani. Plasmid pSV2CAT, described by Gorman et al. (1982) was obtained from American Type Culture Collection (No. 37155).

Analysis of RNA

Polyadenylated RNA was isolated by oligo(dT) chromatography from cell lysates (Schwab et al., 1983). 4–8 μg aliquots of RNA were electrophoresed in 1.2% formaldehyde-agarose gels, transferred to Biodyne nylon filters in 20 × SSC and hybridized with nick-translated probes as described earlier (Sistonen et al., 1987).

Assay of 2-Deoxyglucose (DOG) Uptake

1–2 $\times 10^5$ cells were grown in DME supplemented with 10% FCS for 24–48 h after which the cells were kept in 1% FCS for 48 h before the analysis of 2-DOG uptake according to Flier et al. (1987). The cells were washed three times with PBS and then incubated in glucose-free DME supplemented with 0.1% BSA and 100 nM 2-DOG for 5 min at 37°C. 0.5 μCi [3H]2-DOG (17 Ci/mmol) was added and the uptake was interrupted 5 min later by the addition of ice-cold PBS containing 0.3 mM phloretin. The cells were lysed in 0.1 M NaOH and neutralized with 0.1 M HCl before measurement of radioactivity by liquid scintillation counting. The 2-DOG uptake was related to the total protein content of the cell lysates, which was measured with a protein assay kit (Bio-Rad Laboratories, Cambridge, MA) using BSA as a standard.

Measurement of ODC Activity

About 2 $\times 10^5$ cells were seeded and grown in DME containing 10% FCS for 48 h before the transfer of the cells into the low-serum medium (0.5% FCS). After a further 48 h incubation, EGF or FCS was added to a final concentration of 5 nM or 10%, respectively. At the times indicated (0, 2, 5, 9, and 24 h), the cells were harvested by scraping and assayed for ornithine decarboxylase (ODC), activity essentially as described earlier (Sistonen et al., 1987).

Metabolic Labeling and Immunoprecipitation

For immunoprecipitation of *neu* and EGF-R proteins, rabbit antisera (Lehväslaiho et al., 1989), and mouse monoclonal antibodies against the EGF-R extracellular domain (RPN.513, Amersham Corp.) were used, respectively. The cells were metabolically labeled for 14–16 h with [35S]methionine (100 μCi/ml). Autophosphorylation of the EGF-R/*neu* receptor was analyzed after treatment of the metabolically labeled cells with 25 nM EGF for 2 min at room temperature after which the cell lysates were immunoprecipitated with affinity-purified antiphosphotyrosine antibodies (kindly provided by Dr. H. Fujio, Osaka University, Osaka, Japan; Seki et al., 1983). For immunoprecipitation, the cells were lysed in 0.1% SDS, 0.5% Triton X-100, 0.5% sodium desoxycholate, 20 mM Tris-HCl, pH 7.5, and sonicated for 1 min at 300 W on ice. The lysates were centrifuged for 30 min at 10,000 rpm at +4°C in a HB-4 rotor in a centrifuge (Sorvall, Newtown, CT), and the supernatant was divided in 1 ml aliquots into which ∼1 μl of antibody was added and allowed to bind at 4°C for 1 h. For blocking experiments, 2 μl of antibody was preincubated with 2 μg of bacterial *neu* protein for 20 h. About 30 μl of a 50% vol/vol solution of protein A-sepharose (Pharmacia Fine Chemicals) was added to the antibody-containing lysates and the tubes were mixed gently for 1 h at 4°C. For immunoprecipitation of the EGF-R with mouse monoclonal antibodies, rabbit anti–mouse immunoglobulin-coated, washed protein A-Sepharose particles were used. The immune complexes were washed five times with the immunoprecipitation buffer, twice with PBS, and once with 20 mM Tris-HCl, pH 7.0, dissolved in the electrophoresis sample buffer containing 2% SDS, 5% β-mercaptoethanol, 10% glycerol, and 50 mM Tris-HCl (pH 6.8), and boiled for 5 min.

For detection of the *jun* and *fos* proteins, the cells were serum-starved in 0.5% FCS for 24 h before either EGF (3 nM) or dialyzed FCS (20%) was added to the methionine-free medium in which the cells were incubated for 30 min before methionine labeling (250 μCi/ml) for 45 min at 37°C. Immunoprecipitation of the *jun* protein was carried out using cell lysates preabsorbed with normal rabbit serum and the rabbit polyclonal antisera PEP-1 and PEP-2 (1:1) produced against synthetic v-*jun* peptides (kindly provided by Dr. Peter K. Vogt; Bohmann et al., 1987). The sheep anti-c-*fos* peptide antibodies were from Cambridge Research Biochemicals Ltd. (Cambridge, UK) (DCP 821). The *neu* and EGF-R proteins were analyzed in a 7.5% SDS-PAGE and *jun* and *fos* proteins in a 10% SDS-PAGE according to the method of Laemmli (1970). After electrophoresis, the gels were fixed in 10% acetic acid, impregnated with Amplify (Amersham Corp.), dried onto filter paper, and fluorographed (X-Omat R film Eastman Kodak Co., Rochester, NY).
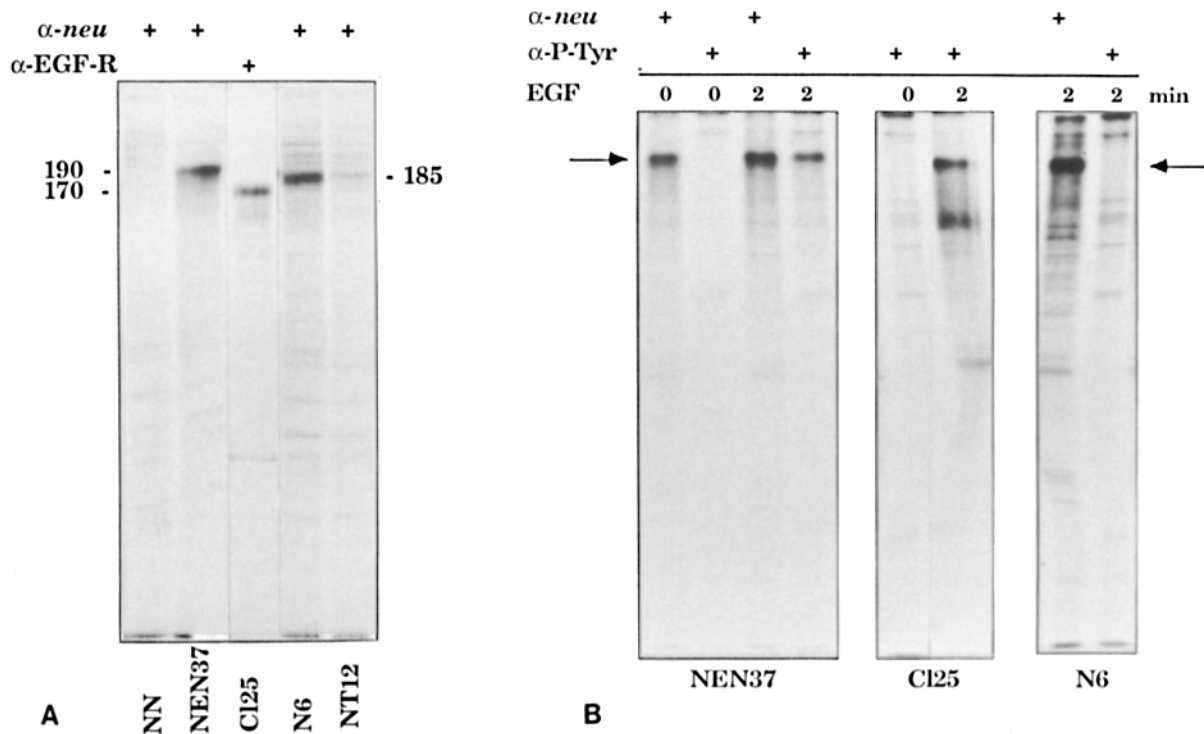
Figure 1. Immunoprecipitation (A) and EGF-induced tyrosine kinase activity (B) of the EGF-R, *neu* and chimeric EGF-R/*neu* growth factor receptors. A, The cells were labeled with [³⁵S]methionine overnight and cell lysates were immunoprecipitated with rabbit antibodies against the carboxyl terminal domain of the rat *neu* receptor expressed in *E. coli* (Lehväslaiho *et al.*, 1989) or with monoclonal anti-EGF-R antibodies as detailed in Materials and Methods. Shown is an autofluorogram of SDS-PAGE analysis of anti–*neu*-precipitates from the NN, NEN37, N6, and NT12 cells as well as anti–EGF-R-precipitates from the Cl25 cells. Note that the *neu* oncogene-transformed NT12 cells express smaller amounts of receptor polypeptides than the other receptor transfectants. B, After metabolic labeling overnight, the cells were stimulated with 25 nM EGF (for 2 min) or with buffer (0) and lysates were precipitated with anti-*neu* and antiphosphotyrosine antibodies, as shown.

Assay for CAT Activity

The cells were seeded at ~4 × 10⁵ cells/100 mm plate and grown in medium containing 10% FCS for 24 h, after which they were changed into medium supplemented with 0.5% FCS and incubated for a further 20 h. The cells were then transfected in low-serum medium with 20 µg of plasmid DNA using the calcium phosphate precipitation method. Incubation with the DNA-precipitate was for 16 h followed by a 1 min rinse in serum-free medium containing 20% glycerol. 24 h later, EGF was added at a final concentration of 10 nM, and after 8 h the cells were harvested and the cell extracts were prepared by freezing and thawing the cells in 100 µl of 0.1 M potassium phosphate (pH 7.8), 1 mM DTT.

The CAT activity was measured essentially as described by Gorman et al. (1982). 60 µl of the cell extract was incubated at 37°C in a final volume of 180 µl together with 0.5 µCi of [¹⁴C]-chloramphenicol. After 30 min, the reaction was stopped and the chloramphenicol was extracted with 1 ml of cold ethyl acetate. ¹⁴C-labeled chloramphenicol was separated from acetylated forms by developing the silica gel thin-layer plates in chloroform-methanol (95:5; ascending). After autoradiography of the separated acetylated chloramphenicol forms, the radioactive spots were cut out and counted. The CAT activity was calculated by determining the percentage of acetylated chloramphenicol per mg protein assayed with a Protein Assay Kit (Bio-Rad Laboratories).

Luciferase Assay

All CAT constructs were cotransfected in ratio of 1:5 with the pRSVL plasmid. Luciferase activity of the cell extracts was analyzed essentially as described by de Wet et al. (1987). 50 µl of 40 mM ATP and 5 µl of 20 mM luciferin were added in 370 µl of 25 mM glycylglycine (pH 7.8) and 15 mM MgSO₄ containing 20 µl of the cell extract. The peak light emission was recorded with a luminometer (1250; LKB Instruments, BioOrbit, Finland).

Results

Autophosphorylation of the EGF-R/neu Receptor upon EGF Stimulation

For the studies on the effects of *neu* tyrosine kinase activation, we used mouse NIH 3T3 cell clones expressing an EGF-R/*neu* hybrid receptor protein on their surface (Lehväslaiho et al., 1989). Fig. 1 A shows immunoprecipitation of the metabolically labeled chimeric receptor from serum-starved cells of the clone NEN37 expressing the chimeric receptor and a comparison with receptor immunoprecipitation from neomycin-resistant control cells (NN), cells expressing the EGF receptor (Cl25), the *neu* proto-oncogene (N6) or oncogene (NT12). Fig. 1 B shows that in NEN37 cells the EGF-R/*neu* chimeric protein was phosphorylated in tyrosine in 2 min after the addition of EGF, similarly to the clones described earlier (Lehväslaiho et al., 1989), while treatment of N6 or NT12 cells had no effect on *neu* protein phosphorylation (Fig. 1 B and data not shown). The EGF-R of the Cl25 cells was also autophosphorylated after EGF-stimulation.

The mRNAs of jun Transcription Factors and the Glucose Transporter Are Induced in Response to EGF Stimulation of the Chimeric EGF-R/neu Receptor

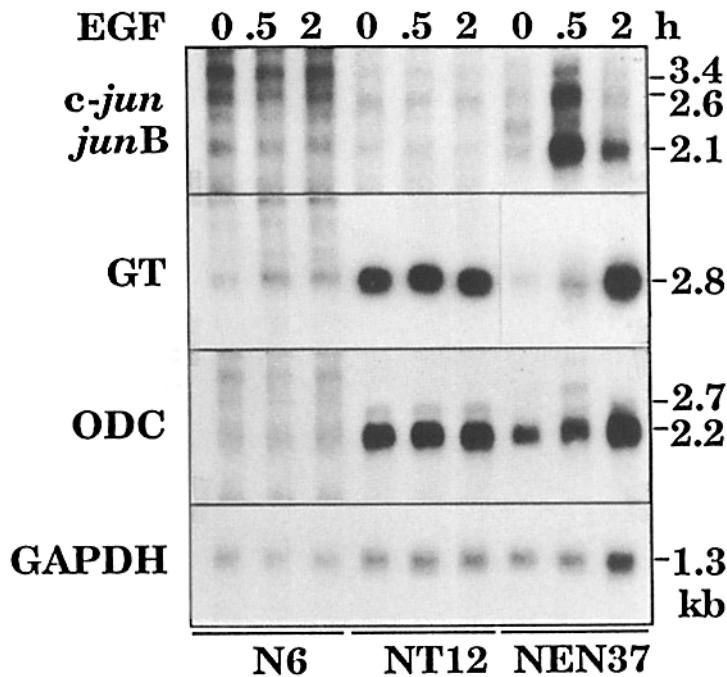We wanted to test whether the *neu* tyrosine kinase was capa-

Figure 2. Induction of early growth factor-stimulated mRNAs by EGF-treatment of cells expressing the chimeric EGF-R/*neu* proto-oncogene. The N6 and NT12 cells expressing the *neu* proto-oncogene and oncogene, respectively, and the NEN37 cells expressing the EGF-R/*neu* construct were treated with 5 nM EGF for the indicated periods of time, lysed, and analyzed in Northern hybridization with the c-*jun*, *jun*B, GT, and ODC cDNA probes, and as a control, with the GAPDH probe. The sizes of the mRNAs are shown on the right in kilobases.

ble of delivering a signal for the growth activation of quiescent cells. To study this response, we analyzed the expression of genes typically activated during the G_0-G_1 and G_1-S phases of the cell cycle. The c-*jun*, *jun*B, and c-*fos* genes belong to the group of genes forming the protein synthesis-independent "immediate early" genomic response to a variety of cellular growth signals (Almendral et al., 1988; Greenberg and Ziff, 1984; Lamph et al., 1988; Lau and Nathans, 1987; Ryder and Nathans, 1988; Ryder et al., 1988; Ryseck et al., 1988). The glucose transporter (GT) mRNA is also induced by growth factors and this induction cannot be prevented by protein synthesis inhibitors (Hiraki et al., 1988; Rollins et al., 1988).

Polyadenylated RNA was isolated at various times after EGF stimulation from the NEN37 cells and hybridized with the c-*jun*, *jun*B, and GT probes. The results are seen in the upper two panels of Fig. 2. The c-*jun* and *jun*B mRNAs were increased about four- and sevenfold after 0.5 h of EGF treat-

ment, and 1.5–2-fold at 2 h. The GT mRNA was about three- and fivefold enhanced at 0.5 h and 2 h of treatment, respectively. In contrast, no increase of either *jun* mRNA or GT mRNA was obtained in N6 cells expressing the *neu* proto-oncogene. However, in NT12 cells the GT mRNA levels, but not *jun* mRNA, were constitutively high regardless of EGF treatment of the cells.

Induction of Ornithine Decarboxylase mRNA

The N6 and NT12 cells also contained constitutively low and high amounts of ODC mRNA, respectively (Fig. 2, *GAPDH*). An EGF-induced increase of ODC mRNA was seen only in the NEN37 cells. The NEN37 cells had somewhat variable uninduced levels of ODC mRNA (depending on the extent of starvation) and showed an about sixfold induction at 2 h (compare Figs. 2 and 3). Examination of the Northern hybridization results of Fig. 3 shows that while the expression
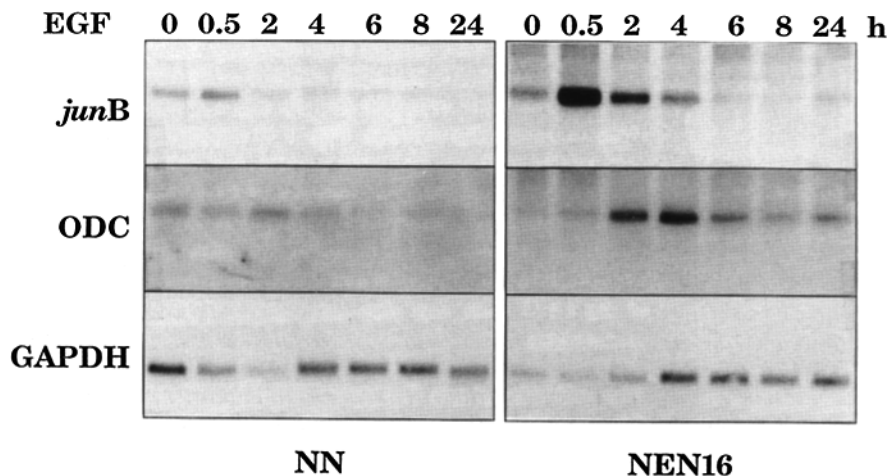

Figure 3. Time course of *jun*B and ODC mRNA induction in the EGF-treated NN and NEN16 cells. The preparation of RNA samples and their analysis with the *jun*B and ODC probes was as shown in Fig. 2. About 8 μg of poly(A)⁺ RNA was loaded per lane. Note that GAPDH mRNA is enhanced beginning 4 h after the addition of EGF.
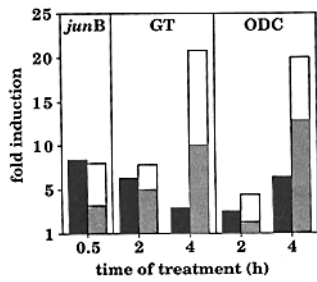
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Figure 4. Effect of cycloheximide on *junB*, GT, and ODC mRNA expression in the NEN37 cells. Growth-arrested NEN37 cells were treated with EGF (5 nM; *black columns*), cycloheximide (10 μg/ml; *grey columns*) or with both EGF and CHX (*white columns*). At the indicated times poly(A)+ RNA was isolated and analyzed by Northern blotting and hybridization with the *junB*, GT, and ODC probes. The autoradiographic signals were quantitated by scanning densitometry and the results expressed as fold of induction over control.

of *junB* was maximal during the first half an hour of EGF treatment and had declined at 6 h back to baseline levels, the expression of ODC mRNA was first enhanced at 2 h of EGF stimulation and reached a maximum (a sixfold increase) at 4 h of EGF treatment in the NEN16 cells (expressing ∼4 × 10⁵ chimeric receptors/cell), returning thereafter slowly to preinduction levels. In contrast, only about a twofold transient induction of *junB* and ODC mRNAs was seen in the neomycin-resistant NIH 3T3 cell clone NN treated with EGF. Also the GAPDH mRNA was slightly enhanced beginning 4 h after EGF treatment.

The Induction of Early mRNAs by the neu Tyrosine Kinase Is Independent of Protein Synthesis

To determine whether the induction of the *junB*, GT, and ODC mRNAs required new protein synthesis we analyzed the effect of cycloheximide (CHX) on the mRNA levels induced by EGF. Serum-starved NEN37 cells were treated with EGF with or without CHX for 0.5, 2, and 4 h. Control experiments were performed with CHX alone. Fig. 4 shows the relative amounts of *junB*, GT, and ODC mRNAs in the cells quantitated by scanning densitometry. CHX slightly increased the maximal EGF-induced GT levels at 2 h and caused a more striking accumulation of the GT mRNA at

4 h, when the mRNA content was returning to basal levels in cells not treated with CHX. CHX also increased the induction of ODC mRNA levels from 2.5- to 4.5-fold at 2 h and from 6- to 20-fold at 4 h, respectively. EGF also increased *junB* mRNA in the presence of CHX, although CHX did not further enhance the EGF-induced response at 0.5 h. However, CHX prevented the downregulation of the *junB* mRNA response at later time points (data not shown).

Increased Synthesis of the Jun and Fos Transcription Factors

The induction of the *jun* and *fos* oncogene-encoded polypeptides was analyzed by labeling the EGF-stimulated cells, and for comparison, serum-stimulated cells with ³⁵S-methionine for 45 min beginning 30 min after the addition of the growth factors and immunoprecipitating cell lysates with the *jun*- and *fos*-specific peptide antisera. As expected on the basis of the results of mRNA analysis of the *neu* oncogene-expressing cells, the biosynthesis of the *jun* protein was not significantly elevated in the *neu* oncogene-expressing NT11 cells and only slightly increased by EGF in the NIH 3T3 cells, NT11 cells or N6 cells expressing the *neu* proto-oncogene (Fig. 5). In contrast, EGF increased the *jun* protein levels of NEN37 cells more than 10-fold, which was of the same order as the stimulation obtained by serum in several experiments. Similar results were obtained for the expression of the *fos* protein, but the *fos* immunoprecipitates also contained several other polypeptides, at least one of which comigrated with the *jun* polypeptides (Fig. 5 *B*). In conclusion, the cells expressing the chimeric receptor are capable of inducing the *fos/jun* protein complex, which is essential for the subsequent activation of genes containing the enhancer consensus sequence for *jun*/AP-1/PEA-1 binding (Chiu et al., 1988).

Activated neu Tyrosine Kinase Is Associated with Increased AP-1/PEA-1 Activity

If the immunoprecipitated *fos/jun* complex is functional after EGF stimulation, promoters containing the consensus AP-1/PEA-1 binding sequence should be activated in the cells
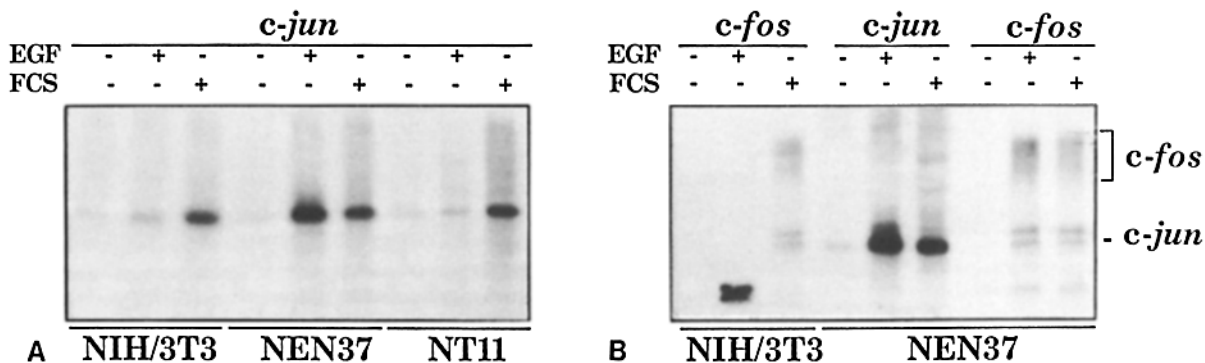

Figure 5. Immunoprecipitation of *jun* and *fos* proteins from EGF-treated cells. Serum-starved NEN37 cells, and as controls, NIH 3T3, and NT11 cells were treated with 10 nM EGF or 20% dialyzed FCS, labeled with [³⁵S]methionine and immunoprecipitated with the rabbit anti-*jun* or anti-*fos* peptide antibodies. Note that FCS induces both *jun* and *fos* proteins in the NIH 3T3 and NT11 cells, while EGF does not. In contrast, EGF induces the *jun* and *fos* proteins more strongly than FCS in the NEN37 cells. Note also that several polypeptides are coprecipitated with the anti-*fos* antibodies and that one of the coprecipitated polypeptides comigrates with the *jun* protein. The black spots in the second lane of the right panel are artefacts of autoradiographic exposure.
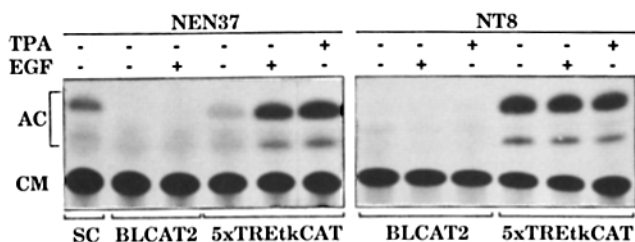
Figure 6. Expression of the 5xTREtkCAT transcription unit is induced by activated *neu*. Serum-starved NEN37 and NT8 cells were transfected with the SV2CAT (*SC*), BLCAT2, or with the 5xTREtkCAT constructs together with pRSVL as described in the Materials and Methods. After 40 h the cells were stimulated with 10 nM EGF or 100nM TPA for 8 h and then analyzed for CAT activity. *CM* and *AC* indicate unacetylated and acetylated forms of the ^{14}C-labeled chloramphenicol, respectively.

(Chiu et al., 1988; Rauscher III et al., 1988). We examined the ability of the *neu* tyrosine kinase to induce transactivation of a recombinant CAT expression vector having an AP-1/ PEA-1-dependent 12-O-tetradecanoyl-phorbol-13-acetate-responsive element (TRE) by using indicator plasmid transfection into the NEN37 cells. Serum-starved cells were transfected either with the plasmid p5xTREtkCAT containing a fivefold TRE element linked upstream of the thymidine kinase promoter and the CAT gene. Alternatively, a similar plasmid pBLCAT2 lacking the TRE sequences was used (Angel et al., 1987). The luciferase plasmid pRSVL was cotransfected with the reporter recombinants pBLCAT2 and p5xTREtkCAT as an internal control for variations in transfection efficiency of different cell clones. 40 h after transfection part of the transfected cells were treated with 10 nM EGF or 100 nM TPA for 8 h, after which cell extracts were prepared and assayed for luciferase and CAT activities (Gorman et al., 1982; de Wet et al., 1987). Similar levels of luciferase activity were obtained from the NEN and NT cells transfected simultaneously with the same DNA precipitate, suggesting that the transfection efficiency did not differ significantly between these cells (data not shown). However, comparisons of CAT activity between the cells were normalized to luciferase activities, where small variation occurred. An ∿2.5-fold increase in CAT activity was obtained in NEN37 cells stimulated either with EGF or TPA, when compared with the CAT activity in untreated cells from the same transfections (Fig. 6). Interestingly, both of the two *neu* oncogene-expressing cells tested (NT8 and NT 12) showed a high basal level of AP-1/PEA-1 activity (about 4-fold enhanced), which was insensitive to both EGF and TPA stimulation (Fig. 6, and data not shown).

Stimulation of Glucose Transport

Stimulation of glucose transport provides a sensitive way of analyzing growth factor responses in cells (Martin et al., 1971). We tested the activity of the glucose transporter by measuring the rate of uptake of 2-DOG into EGF-treated cells. The cells were growth-arrested by placing the nearly confluent cultures in medium supplemented with 1% FCS for 48 h, after which the cells were treated either with EGF or dialyzed FCS. After a 2 h exposure to EGF, the NEN37 cells expressing the EGF-R/*neu* receptors showed markedly

increased rates of 2-DOG uptake (two- to threefold increase during the first 8 h), whereas the EGF-treated N6 and NT11 cells displayed very little change of 2-DOG transport throughout the experiment (Fig. 7). In the C125 cells, which express similar levels of EGF receptors, the 2-DOG uptake rate was linearly increased for up to threefold after 8 h treatment with EGF or serum. The uptake was stimulated by serum also in the other cell clones, although the induction was only about twofold in *neu*-oncogene expressing NT11 cells, which showed a constitutively accelerated rate of glucose transport (Fig. 7, legend), characteristic for oncogenically transformed cells (Flier et al., 1987).

ODC Is Stimulated by the Activated neu Tyrosine Kinase

Measurement of ODC activity from cell lysates after various periods of EGF stimulation showed that while the uninduced levels of ODC activity were similarly reduced in starved NEN37 cells expressing the ECF-R/*neu* chimera and in control cells, EGF increased ODC activity considerably only in the EGF-R/*neu* receptor-expressing cells (Fig. 8). However, a very small and transient increase of the ODC activity was also observed in the EGF-treated control cells. This increase is not apparent in Fig. 8 because of the scale of the ODC activity. In NEN37 cells that were starved for serum for 2 d the activity of ODC was ∿60-fold increased (from 0.09 to 5.4 nmol/mg protein/h) 2 h after EGF stimulation and further stimulated 70-fold (from 0.1 to 6.8 nmol/mg protein/h) and 300-fold (from 0.09 to 26.4 nmol/mg protein/h) at 9 h and 24 h, respectively. When the serum-starvation of cells was reduced to 24 h, the EGF-induction of ODC followed a much more rapid kinetics, with the maximal activity level reached
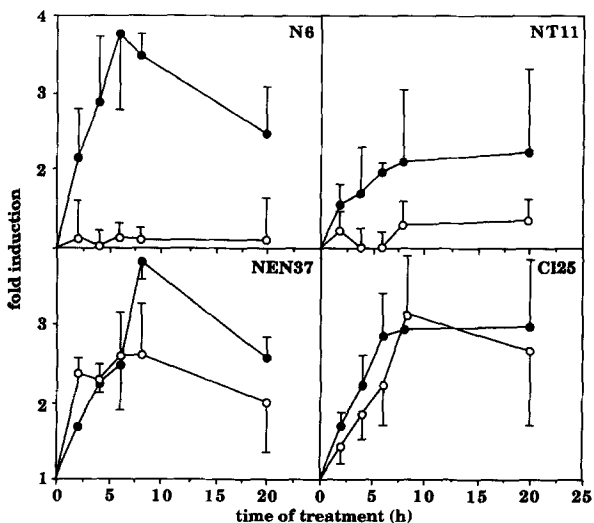

Figure 7. Effects of EGF and serum on 2-DOG uptake. About 80% confluent cultures of N6, NT11, NEN37, and C125 cells were incubated in DME supplemented with 1% FCS for 48 h. EGF or dialyzed FCS was added to final concentrations of 10 nM or 10%, respectively. After the indicated times the uptake of 2-DOG was measured as described in Materials and Methods. Each value (open circles for EGF treatment, closed circles for serum treatment) represents the mean value of at least two separate experiments. At the time point zero the following rates of 2-DOG uptake were obtained: N6 cells, 54 cpm/5 min/µg prot.; NT11 cells, 158 cpm/5 min/µg prot.; NEN37 cells, 35 cpm/5 min/µg prot.
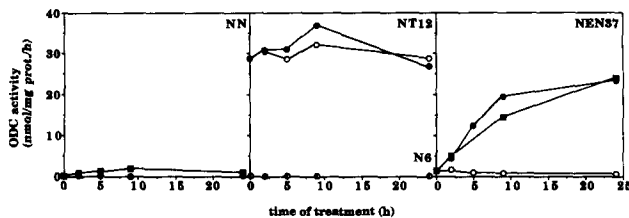
Figure 8. Kinetics of ODC activity in serum-starved cells treated either with 5 nM EGF or 10% serum. Open and closed circles show the ODC activities of the untreated and EGF-treated NN, N6, NT12, and NEN37 cells, respectively. Serum-induced ODC activities of the NN cells and the NEN37 cells are indicated with closed squares.

as early as in 2 h (data not shown). Similar results were obtained with the other cell clones. (NEN7, NEN16, and NEN49) expressing the chimeric receptor (data not shown). It is also notable that the increase in ODC activity upon EGF treatment of NEN37 cells was approximately of the same magnitude as was obtained with serum stimulation. The ODC activity obtained in NN cells after serum stimulation was higher than that obtained with EGF but still much below the induced ODC activities in NEN37 cells. Thus, the maximal ODC activity measured in EGF-stimulated chimeric EGF-R/*neu* cells was several 100-fold the activity measured from the EGF-stimulated neomycin-resistant control cells or from the N6 cells expressing the proto-oncogene. In contrast, the NT12 cells expressing the *neu* oncogene had ~500-fold elevated, EGF-independent ODC activity (Fig. 8).

Discussion

Our results show that the activated *neu* tyrosine kinase induces changes of transcriptional, nutrient transport and enzymatic activities typical for cell growth activation and cell cycle progression of growth factor-stimulated cells. These effects mimic those induced by EGF in EGF-receptor expressing cells (DiPasquale et al., 1978; Quantin and Breathnach, 1988; Hiraki et al., 1988). One of the earliest changes in gene expression induced by the activation of the *neu* tyrosine kinase is the induction of the mRNA and protein for the *jun* transcription factors, followed by an increase in the expression of the GT and ODC mRNAs. Interestingly, all three mRNAs were induced in the presence of the protein synthesis inhibitor CHX, indicating that these genes are stimulated by preexisting factors in the responding cells. Possibly, such factors become modified by posttranslational mechanisms, such as protein phosphorylation and become capable of stimulating the transcription of the set of immediate early and early responding genes.

Our immunoprecipitation results indicated that cells of the NEN37 clone expressing the chimeric EGF-R/*neu* protein at ~4 × 10⁵ receptors/cell as well as cells of the clone Cl25 expressing the EGF-R at similar levels reacted to EGF stimulation by a rapid tyrosine phosphorylation of the receptors. In contrast, EGF had no effect on the antiphosphotyrosine immunoprecipitation of *neu* proteins from cells of the N6 or NT 12 clones expressing the *neu* proto-oncogene and oncogene, respectively. These results establish that the cells clones used in this study express the EGF-R/*neu* tyrosine

kinase and EGF-R kinase activities that are controlled by ligand binding and that the *neu* proto-oncogene and oncogene-encoded kinases are not affected by EGF in the control cell clones.

In NIH 3T3 cells expressing the chimeric EGF-R/*neu* receptor the *jun* transcription factor mRNAs are enhanced within 30 min after addition of EGF. These genes, *jun*B and c-*jun* encode protein components of the transcription factor AP-1/PEA-1 (see Curran and Franza, 1988). The EGF-treated cells contain the typical *fos*/*jun* transcription factor complex as shown by metabolic labeling and immunoprecipitation ~1 h after stimulation. This complex can also be seen at similar elevated levels in serum-stimulated NEN cells and control cells, but it is not precipitated from the EGF-treated control cells, including cells transformed by the *neu* oncogene.

The *fos*/*jun* complex has been shown to bind tightly to the TRE enhancer DNA elements and to activate transcription of promoters under their influence (Angel et al., 1987, 1988a; Chiu et al., 1988). Interestingly, the *fos*/*jun* complex has also autostimulatory and inhibitory properties on the expression of the c-*fos* and c-*jun* genes themselves (Angel et al., 1988b; Sassone-Corsi et al., 1988). We show evidence for an increased AP-1/PEA-1 transcription factor activity in the EGF-treated cells expressing the chimeric receptor by use of a recombinant 5xTRE-CAT vector and transient expression assays. The effect of EGF measured at 8 h of stimulation was comparable to the degree of induction by TPA and to the activity present in unstimulated *neu* oncogene-expressing cells. It is possible that the increase in the amount of the *fos*/*jun* complex, induced by the ligand activation of the *neu* tyrosine kinase, is capable of activating the subsequent set of genes, whose expression is dependent on the synthesis of novel proteins and can therefore be classified as "early" growth factor-induced genes. However, the reason for the high constitutive activity of a TRE element in transformed cells remains unknown. Our experiments with the *ras* and *neu* oncogenes suggest that elevated *jun* and *fos* mRNA and protein expression may not be responsible for this difference (Sistonen et al., 1989; our present study). Possibly, the *fos* protein might be modified by constitutive posttranslational phosphorylation subsequent to the mutational activation of the *neu* tyrosine kinase and the modified *fos*/*jun* complex then could maintain several genomic responses. On the other hand, we have not yet analyzed the possibility that these proteins would be more stable in the transformed cells.

Since an accelerated rate of glucose transport is among the most characteristic biochemical markers of growth stimulation and the transformed phenotype, we decided to use the analysis of the GT gene expression to study the pattern and kinetics of gene activation in our EGF dependent growth and transformation model system. cDNAs encoding the human hepatoma (Mueckler et al., 1985) and the rat brain (Birnbaum et al., 1986) GT have been used to demonstrate that elevated levels of GT mRNA are associated with an accelerated rate of glucose transport in oncogenically transformed cells (Birnbaum et al., 1987). In the case of cells transformed with v-*src* or activated c-*ras* oncogenes, it was suggested that the signal transduction mechanisms activated by the oncogene would be associated with the elevated GT mRNA expression (Flier et al., 1987). In accordance with these results, the increased rate of glucose transport in the *neu*

oncogene-transformed NT cells correlated with significantly elevated GT mRNA levels.

Recently, it has also been shown that serum and the purified growth factors EGF, PDGF and FGF regulate the glucose transport system at a transcriptional level (Hiraki et al., 1988; Rollins et al., 1988). The GT mRNA induction in the NEN cells expressing the EGF-R/neu receptor was independent of protein synthesis, as has been found using other cells and EGF and PDGF receptor-ligand systems (Hiraki et al., 1988; Rollins et al., 1988). The time course of EGF-induced GT mRNA increase correlates with the actual increase of glucose transport measured by the uptake of 2-DOG, although the mRNA levels were already declining at 4 h of treatment, when the 2-DOG transport rate was still rising. The maximal enhancement of the GT mRNA was about sixfold, whereas the maximal transport rate was threefold over unstimulated values. This parallels the results of Rollins et al. (1988) and supports the hypothesis on the existence of translational and posttranslational controls of GT expression.

We have previously shown that in NIH 3T3 cells, where expression of the activated c-Ha-ras oncogene is induced from the human heat shock promoter 70 the onset of synthesis of p21$^{c-Ha-ras}$ oncoprotein is rapidly followed by elevated mRNA levels of the junB, c-jun, GT and ODC genes (Sistonen et al., 1989). Our present results show that the neu tyrosine kinase is also capable of enhancing the expression of these mRNAs, with jun mRNA rising first and the GT and ODC mRNAs thereafter. It has previously been shown that the mitogenic induction of ODC mRNA results, at least partly, from increased transcription of the ODC gene (Katz and Kahana, 1987). The ODC activity follows these changes, but the timing of the peak of ODC activity after EGF stimulation is dependent on the length of prior cell starvation, the dosage of EGF, and the number of receptors on the responding cells. It is of interest that the maximal ODC activities stimulated by EGF and serum in the NEN37 cells are of similar magnitude and closely correspond to the constitutive ODC activity of the neu oncogene-transformed cells. Further, the induction of ODC by EGF in the chimeric EGF-R/neu expressing cells precedes by several hours their morphological transformation. All these results are consistent with the view that ODC is expressed during the G_1 period of the cell cycle, and that the length of the lag period depends on the state of the cells in the G_1/G_0 phase of the cell cycle before cell stimulation. Thus, this study further strengthens our earlier suggestions based on the c-Ha-ras oncogene-transformed cells (Sistonen et al., 1987; Höltta et al., 1988) that a deregulated expression of ODC is intimately associated with cell transformation and that a high constitutive activity of ODC is typical for stably transformed cells.

The neu oncogene is activated by a point mutation exchanging glutamic acid for valine in the transmembrane domain of the receptor (Bargmann et al., 1986b). However, it is not known how the neu oncoprotein transforms cells. Enhanced tyrosine kinase activity and an increased phosphotyrosine content have been reported for the neu oncoprotein in rat cells (Bargmann and Weinberg, 1988a,b; Stern et al., 1986; 1988). The half-life of the oncoprotein is also shorter than the half-life of the proto-oncogene-encoded neu protein (Stern et al., 1988), suggesting that the oncoprotein undergoes a more rapid rate of internalization and is also targeted to enhanced degradation in lysosomes as proposed for the

EGF-R (Schlessinger, 1988). This view is consistent with the recent model for the effects of the activating point mutation of the neu oncoprotein, which may greatly increase the rate of receptor dimerization, normally occurring only after ligand stimulation of the neu proto-oncogene-encoded receptor (Sternberg and Gullick, 1989). Our chimeric EGF-R/neu receptor allows at least a tentative model for comparison of the biochemistry and genomic effects of the ligand- and oncogenically activated neu receptors. The present study indicates that both types of receptor activation lead to similar biochemical changes typical for growth factor-stimulated and transformed cells. Interesting differences also exist, as is shown here for the activation of the fos/jun transcription factor complex in the presence and absence of an increase of the corresponding protein components in ligand-stimulated and constitutively neu-transformed cells. The further analysis of such differences will undoubtedly shed light on the pathways of growth factor signal transduction and their derangements in transformed cells.

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