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Quantitative Dissociation Between EGF Effects on *c-myc* and *c-fos* Gene Expression, DNA Synthesis, and Epidermal Growth Factor Receptor Tyrosine Kinase Activity

SYLVIE HAUGUEL-DeMOUZON, PETER CSERMELY, GIACOMO ZOPPINI, AND C. RONALD KAHN*

Research Division, Joslin Diabetes Center, Department of Medicine, Harvard Medical School, and Brigham and Women's Hospital, Boston, Massachusetts 02215

The exact relationship between EGF-stimulated tyrosine phosphorylation, induction of the cellular proto-oncogenes *c-myc* and *c-fos*, and DNA synthesis remains uncertain. Madin-Darby Canine Kidney (MDCK) cells possess EGF receptor sites with high binding capacity, and in contrast to A431 cells, respond to EGF by increasing DNA synthesis. Following EGF stimulation of intact MDCK cells, there was a rapid and marked increase in the autophosphorylation of the EGF receptor. This was associated with an increase in the tyrosine phosphorylation of a 120 kDa phosphoprotein believed to be an endogenous substrate of this receptor kinase. The ED_{50} for stimulation of phosphorylation of pp120 was ~0.05 nM versus 1.0 nM for receptor autophosphorylation, consistent with amplification of signalling at this step in EGF action. Stimulation of DNA synthesis occurred after 12 to 24 hours and revealed even further amplification with an ED_{50} of about 0.1 nM. Intermediate between these events was a time-dependent activation of *c-fos* and *c-myc* gene expression. However, the ED_{50} for these processes was ~10 nM, indicating a relatively lower sensitivity of EGF for stimulation of proto-oncogene expression. Tyrphostin (RG 50864), a compound reported to inhibit specifically the EGF receptor kinase, completely blocked EGF stimulation of proto-oncogene induction. Interestingly, under the same experimental conditions, EGF receptor autophosphorylation was decreased only 60%. These data, along with the dose-response studies, indicate that proto-oncogene induction requires near maximal stimulation of EGF receptor autophosphorylation. They also suggest that, in MDCK cells, the EGF dependent induction of the *c-fos* and *c-myc* genes is not strictly correlated to the extent of EGF receptor autophosphorylation or EGF-stimulated DNA synthesis, and that EGF stimulation of DNA synthesis likely involves additional rate-limiting intermediate steps.

Epidermal growth factor (EGF) is a potent mitogen for a variety of cells both in vivo and in vitro (Carpenter and Cohen, 1979; Maratos-Flier et al., 1987; Boni-Schnetzler and Pilch, 1987; Schlessinger, 1988; Aharonov et al., 1978; L'Allemain and Pouyssegur, 1986; Moolenar et al., 1988). From a molecular point of view, however, the EGF receptor and the immediate post-receptor responses have been best studied in A431 cells in which EGF is an inhibitor of growth and proliferation (MacLeod et al., 1986). As with other growth factors, the biological activity of EGF can be divided into early and delayed responses. The short-term bio-effects of EGF are measurable within seconds or minutes and include effects on calcium flux (Macara, 1986), activation of phospholipid metabolism (L'Allemain and Pouyssegur, 1986), and stimulation of the expression of a set of cellular genes called "immediate-early" genes (Lau and Nathans, 1987). Two of these genes are the nuclear proto-oncogenes *c-fos* and *c-myc* which are activated by EGF and a variety of other polypeptide growth factors (Muller, 1985; Muller et al., 1984; Forsthoefel and Thompson, 1987; Hall et al., 1987) and have been associated with stimulation of cell proliferation by hormones and serum (Muller et al., 1984; Greenberg and Ziff, 1984). Induction of these genes is believed to function as a signal in mediating the mitogenic response of these growth factors. Stimulation of DNA synthesis in cultured cells is regarded as a delayed effect, since it starts several hours after EGF treatment and peaks at 24 h.

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*To whom reprint requests/correspondence should be addressed.

Sylvie Hauguel-deMouzon's current address is INSERM U 145, Nice, France.

Peter Csermely is on leave from the Institute of Biochemistry I, Semmelweis University, Medical School, Budapest, Hungary.

Thus far it is still a matter of speculation whether or not stimulation of proto-oncogene expression is required for DNA synthesis or regulation of other metabolic functions (i.e., enzyme activity or induction of other genes) in the course of a normal cell cycle. In addition, the exact link between EGF receptor tyrosine kinase activation and the induction of EGF-regulated genes has not been elucidated. The present work was aimed at investigating the relationship between *c-fos* and *c-myc* gene induction, EGF-stimulated receptor kinase activity and associated substrate phosphorylation, and DNA synthesis. We find that induction of the *c-myc* and *c-fos* by EGF is a rather insensitive step in the EGF action cascade and may be blocked by inhibitors which only partially inhibit receptor and substrate phosphorylation, suggesting that the induction of these proto-oncogenes requires near maximal receptor signalling and, thus, is not tightly linked to EGF stimulation of DNA synthesis.

MATERIALS AND METHODS

Materials

Tissue culture plasticware was from NUNC (Kamstrup, Denmark). General tissue culture media and stock solutions were from Gibco (Grand Island, NY). Formaldehyde and phenol were from Fisher Scientific (Pittsburgh, PA). Formamide was from E-M Science (Cherry Hill, NJ). ^{3}[H]-thymidine was purchased from Amersham (Burkinghamshire, England). IGF-I[1] and IGF-II were generously supplied by Dr. R. DiMarchi, Eli Lilly Research Laboratories (Indianapolis, IN).

Anti-phosphotyrosine antibodies were kindly provided by Dr. Morris F. White (Joslin Diabetes Center). EGF was purchased from Collaborative Research (Lexington, MA). The EGF-receptor tyrosine kinase inhibitor, RG 50864 (tyrphostin), was a kind gift of Drs. J. Schlessinger and Alan Schreiber (Rorer Biotechnology Inc., King of Prussia, PA). Protein A acrylamide beads were from Pierce (Rockford, IL). ^{125}I protein A (200 kBq/μg) was an ICN product (Irvine, CA). The chemicals used for gel electrophoresis were from Bio-Rad; nitrocellulose (0.45 μm pore size) was from Schleicher & Schuell (Keene, NH). All the other reagents (aprotinin, agarose, salmon sperm DNA, EGF, DTT, EDTA, Hepes, sodium molybdate, sodium vanadate, PMSF, and TCA) were from Sigma (St. Louis, MO).

Cell culture

Madin-Darby Canine Kidney (MDCK) cells were obtained from the American Type Tissue Collection (Rockville, MD) and grown to confluence on 80 cm^2 culture flasks in Dulbecco's Modified Eagles medium (MEM) containing 5% fetal calf serum. Cells were plated on 10 cm culture dishes and fed by changing medium every other day. At 3 to 5 days after plating, just before reaching confluence, the medium was aspirated, and the cells were starved overnight in serum-free medium in preparation for stimulation experiments by EGF. In some experiments, the EGF-receptor tyrosine kinase inhibitor RG 50864 or its vehicle (DMSO) were added to medium containing 0.1–0.5% fetal calf serum for 20 h prior to the stimulation with EGF, or as indicated.

EGF binding studies

MDCK cells were plated directly into 35 mm multiwell dishes. After 2 days, the medium was changed to serum-free MEM overnight. The following morning, the cells were washed twice with cold PBS. ^{125}I-labeled EGF (25,000 cpm/ml) in binding buffer (Ham's F-12, 25 mM Hepes, and 0.1% albumin, pH 7.8) was added to each well with or without 10 μM unlabeled EGF in a final volume of 1 ml. After a 3 h incubation at 15°C, the radioactive medium was removed, and the wells were washed twice with cold PBS. The cells were solubilized in 1 ml 0.1% SDS, and the amount of ^{125}I radioactivity recovered from each well was counted.

Isolation and analysis of phosphotyrosine containing proteins from MDCK cells

MDCK cells were grown to confluence in MEM medium supplemented with 5% FCS, and serum stained 24 hours prior to the experiment. ^{32}P-phosphate (1 mCi/ml), RG 50864 (Tyrphostin), and EGF were added at times and final concentration indicated. The cells were washed two times with chilled PBS and harvested in a boiling buffer containing 150 mM Hepes (pH 7.5), 200 mM NaCl, 4 mM EDTA, 100 mM DTT, and 2% (w/v) SDS. Proteins were precipitated with 10% TCA, washed with ethanol and diethyl ether, dried, and resuspended in 0.1 N NaOH. A threefold excess of buffer containing 200 mM Hepes (pH 7.0), 2 mM EDTA, 2 mM PMSF, 20 μg/ml aprotinin, 1 mM sodium vanadate, and 1 mM sodium molybdate was added to the resuspended precipitate, and the proteins containing phosphotyrosine were immunoprecipitated with antiphosphotyrosine antibodies (concentration = 10 μg/ml) and protein A acrylamide beads. The immunoprecipitates were washed and subjected to SDS-PAGE as described below. The gels were immunoblotted on nitrocellulose paper and probed with antiphosphotyrosine antibodies (2 μg/ml) and ^{125}I-protein A (1 μCi/ml) for 2 hours at 15°. After extensive washing with 20 mM Tris (pH 7.4) 0.15 M NaCl, 0.5% Tween 20 (U/V), and 0.1% bovine serum albumin, the phosphotyrosine-containing proteins were visualized by autoradiography.

For some experiments, cells were incubated with 0.5 mCi/ml of ^{32}P-orthophosphate for 2 hours prior to EGF stimulation. The reaction was stopped by washing the cells in ice-cold buffer and solubilized in 1% Triton X-100. The phosphoproteins were then precipitated with anti-phosphotyrosine antibody and separated under reducing conditions on 7.5% polyacrylamide resolving gels. Samples were prepared in 2% SDS containing 10% glycerol and 100 mM DTT and heated in boiling water for 3 min prior to electrophoresis. Following

[1]Abbreviations used: DMSO, dimethylsulfoxide; DTT, dithiothreitol; EGF, epidermal growth factor; FCS, fetal calf serum; Hepes, 4-(2-hydroxyethyl)-1-piperazine ethanesulfonic acid; IGF, insulin-like growth factor; MDCK, Madin Darby Canine Kidney; MEM, Modified Eagle's media; Na_3VO_4, sodium vanadate; PBS, phosphate-buffered saline; PMSF, phenylmethylsulfonyl fluoride; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; TCA, trichloroacetic acid; WGA, wheat germ agglutinin.

separation, the positions of radioactively labeled proteins were determined by autoradiography of fixed and dried gels. Molecular weights were estimated by comparison to known standards. The intensity of the signals shown on the autoradiograms was quantified by scanning densitometry.

RNA isolation and quantitation

Cells grown in monolayer and subjected to EGF stimulation for the indicated time were rinsed twice with cold PBS and solubilized directly with 4 M guandium isothiocynate containing 0.75 M citric acid (pH 7.4), 10% N-lauryl-sarcosine, and 0.72% β-mercaptoethanol. Total cellular RNA was isolated by repeated low temperature 4 M guanidium thiocyanate-phenol-chloroform extraction and cold ethanol precipitation (Chomczynski and Sacchi, 1987). After separation in a denaturing 1% agarose, 2.2 M formaldehyde gel, RNA was transferred by capillary action onto nitrocellulose membranes. The membranes were air dried and baked under vacuum at 80°C for 2 h. A 2.8 kb DNA fragment coding for the third exon of human *myc* and a 1.5 kb DNA fragment of the mouse *fos* gene were purchased from ONCOR (Gaithersburg, MD). Human β-actin was a 1 kb insert from Clontech (Palo Alto, CA). After denaturation, the cDNA probes were labeled with [α^{32}P]dCTP (New England Nuclear, Billerica, MA) to approximately 10^9 dpm/μg using a random hexamer priming kit (Prime Time, International Biotechnology, New Haven, CT). Unincorporated label was separated from labeled DNA using an Elutip-d column (Schleicher and Schuell, Keene, NH), and the specifically labeled DNA fragment was eluted using a high salt concentrated solution.

Prehybridization was performed at 42°C for 2 h in a solution containing 4× SSC and 40% formamide followed by hybridization for 16 h at 42°C after addition of the radiolabeled probe into the same solution. At the end of the hybridization period, the filter was washed 4 times for 60 min at room temperature in 1× SSC with 0.1% SDS. This was followed by a fifth wash at 55°C in 0.1× SSC with 0.01% SDS. After autoradiography with an intensifying screen at −70°C for 48 to 96 hours, scanning densitometry of autoradiograms was used to quantify the relative amounts of mRNA species.

Thymidine incorporation into DNA

Confluent cells were trypsinized and plated at a density of 20×10^4 per well in 16 mm multi-well trays (NUNC) in MEM containing 5% fetal calf serum. Four days after plating, medium was replaced with serum-free MEM for 24 hours. EGF (0 to 10 mM) was added to each well for 18 to 24 hours after which 2 μCi/ml of ^{3}[H]-thymidine was added, and the incubation was continued for an additional 4 h. The cells were washed twice with ice-cold phosphate-buffered saline (PBS) and solubilized with 0.1% SDS at 37°C for 30 min. Then 800 μl of the solubilized cell solution was added to 100 μl of 100% trichloroacetic acid (TCA), and the TCA precipitable material was collected by filtration on Whatman filters prewet with 10% TCA. The dried filters were transferred to 3 ml of scintillant and counted in a liquid scintillation β counter.

Cell number and protein determination

Cells were plated at a density of 20×10^4 per well in 16 mm multi-well dishes, grown to confluency, starved for 20 h in serum-free MEM, and finally incubated for 24 h at 37°C in serum-free MEM with 10 nM and without EGF. Cells were then detached with trypsin and counted in a Coulter Counter. Protein concentration of solubilized cells was determined using the Bio-Rad assay and IgG as a standard (Bradford, 1976).

RESULTS

Effects of EGF on DNA synthesis and cell growth

EGF increased ^{3}[H]-thymidine incorporation into DNA and growth of MDCK cells as assessed by an increase in total cell number (Fig. 1A,B). Culture of MDCK cells in the presence of 10 nM EGF led to a 41% increase in cell number and a twofold augmentation of DNA synthesis over 24 hours as compared with cells grown in the absence of EGF. Stimulation of thymidine incorporation into DNA revealed a dose-dependent relationship with some effect at EGF concentrations as low as 0.01 nM (Fig. 1A). To investigate the early cellular events leading from EGF receptor binding to this mitogenic event, we analyzed EGF-receptor tyrosine kinase activity, substrate phosphorylation, and the induction of the "immediate-early" nuclear proto-oncogenes, *c-myc* and *c-fos*.

EGF receptor kinase activity

After binding, EGF is known to rapidly stimulate the intrinsic tyrosine kinase activity of its own receptors leading to receptor autophosphorylation. In MDCK cells, receptor autophosphorylation was assessed by labeling the cells with ^{32}P-phosphate, precipitation of tyrosine phosphorylated proteins contained in whole cell extracts using anti-phosphotyrosine antibody, and analysis of these proteins by SDS-PAGE and autoradiography. EGF induced a time- (Fig. 2A,C) and dose-dependent (Fig. 2B) increase in EGF receptor autophosphorylation with a twentyfold increase over basal level at 10 nM ligand. The phosphorylated EGF receptor was present as a single band with a M_r = 170,000. In addition, EGF stimulated the phosphorylation of a M_r = 120,000 protein (Fig. 2A,B). This is an abundant phosphotyrosine-containing protein (Izumi et al., 1987; Wanen et al., 1988) and is the predominant phosphotyrosine containing protein of MDCK cells in the basal state (Hauguel deMouzon and Kahn, 1991). Phosphorylation of pp120 displayed a similar time-dependent increase as the EGF receptor, although the maximal stimulation of phosphorylation was only about four- to fivefold. EGF also stimulated phosphorylation of three minor protein bands at M_r ~97 kDa, 70 kDa, and 45 kDa.

Induction of *c-myc* and *c-fos* gene expression by EGF

Although EGF receptor tyrosine kinase activity has been associated with nuclear oncogene induction in other cell lines (Cutry et al., 1989; Lindsten et al., 1988), the specific pattern of induction is poorly documented. In MDCK cells, both *c-myc* and *c-fos* mRNA, as quantified by Northern analysis, were apparent even

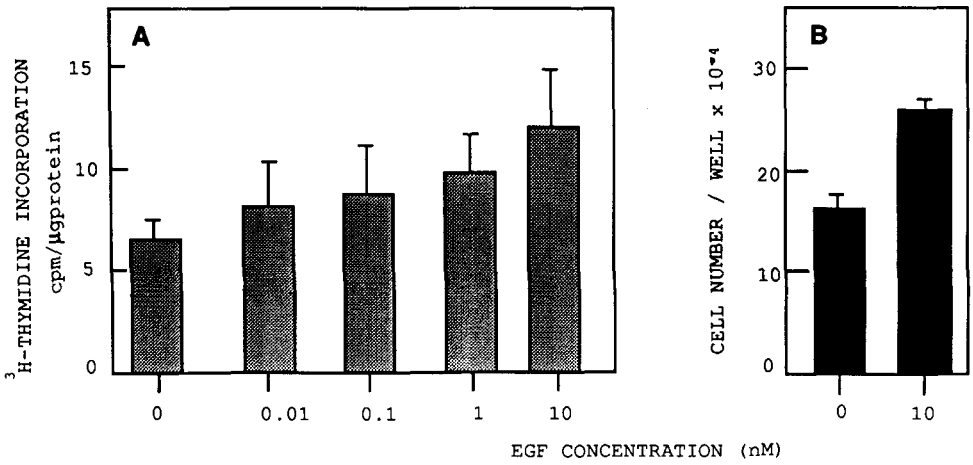

Fig. 1. Effect of EGF on DNA synthesis and cell number. ^{3}[H]-thymidine incorporation (**A**) was measured on cells plated in 16 mm multi-well trays and fed for 4 days with MEM containing 5% FCS. Prior to the experiment, the cells were incubated for 24 h in serum-free medium and stimulated for another 18–24 h with EGF at the indicated concentrations. ^{3}[H]-thymidine (2 µCi/ml) was then added for 4 h and the TCA insoluble radioactivity was determined. Cell number (**B**) was measured after starving the confluent cells for 24 h in serum-free medium. Cells were then incubated for another 24 h without or with 10 nM EGF. At the end of the incubation, the cells were detached with trypsin and counted. The number of cells per mg of protein are plotted against the EGF concentration used for stimulation. All data represent the mean ± SE of 3–4 experiments.

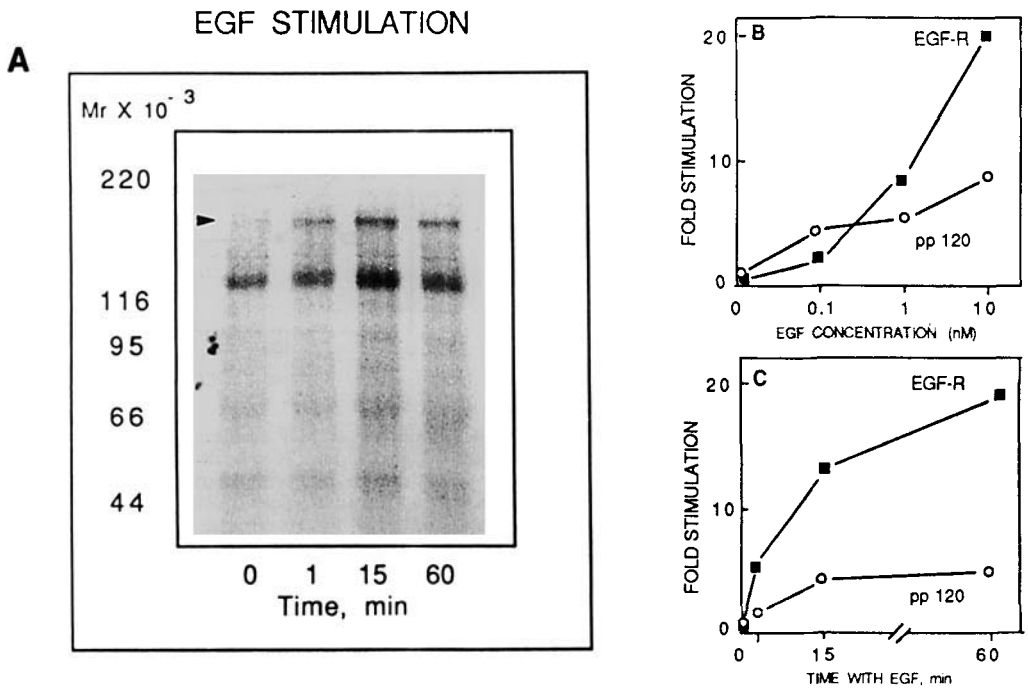

Fig. 2. EGF-dependent tyrosine phosphorylation of EGF-R (170 kDa) and 120 kDa protein in living cells. MDCK cells were incubated for 2 h at 37°C in humidified air containing (5% CO_2) in phosphate-free medium containing 0.5 mCi/ml ^{32}P-orthophosphate, followed by stimulation with 0 to 10 nM EGF for the indicated time intervals under the same incubation conditions. The cells were then solubilized in 1% Triton X-100 and centrifuged to remove insoluble material. The phosphorylated proteins were immunoprecipitated from the supernatants using 2 µg/ml anti-phosphotyrosine antibody and separated by SDS-PAGE under reducing conditions on 7.5% resolving gels. **A:** Autoradiogram of a representative time-course experiment. **B:** Scanning densitometry analysis of a dose-response experiment. After ^{32}P labeling, the intact cells were stimulated for 30 min with the indicated concentrations of EGF. **C:** Scanning densitometry data obtained from the quantification of the autoradiograms similar to that presented in A. After labeling, the cells were stimulated with 10 nM EGF for the indicated time intervals.

in the absence of growth factor or serum stimulation, although the basal level of *c-fos* expression was generally lower than that of *c-myc*. EGF was a very potent stimulus of *c-fos* gene expression producing a fifteen- to twentyfold stimulation of *c-fos* mRNA levels. These peaked at 30 min and returned to basal between 1 and 4 hours. *c-myc* showed a similar early response, but did not show the rapid fall of *c-fos* (Fig. 3). In addition, maximal *c-myc* mRNA expression was only fivefold over basal, but this was sustained for more than 1 hour. Increasing the EGF concentration up to 100 nM was able to further stimulate *c-fos* expression up to fortyfold basal levels, but had no further effect on *c-myc* levels (data not shown). It is also of note that the maximal stimulation of oncogene expression was achieved prior to maximal activation of EGF receptor autophosphorylation—that is, the peak of *c-fos* was at 15 min, whereas the peak of phosphorylation occurred at 60 min (Figs. 2C, 3).

Relationship between EGF receptor kinase activity and EGF-induced biological effects

To explore the role of EGF receptor tyrosine kinase activity on intracellular signal transduction, we used a specific EGF receptor kinase inhibitor in attempt to block these receptor-induced cellular responses. The antiproliferative agent tyrphostin (RG 50864) has been shown to exert its biological effect by inhibiting cell growth and proliferation by blocking selectively the kinase activity of the EGF receptor (Lyall et al., 1989; Margolis et al., 1989). Preincubation of MDCK with tyrphostin produced a dose-dependent inhibition of EGF-stimulated tyrosine phosphorylation (Fig. 4). At a concentration of 100 μM, there was a 60% reduction in EGF-stimulated receptor autophosphorylation. Increasing the tyrphostin concentration to 250 μM did not produce any further inhibition. At 300 μM, however, tyrphostin further reduced the phosphorylation of both the EGF receptor and other EGF-stimulated proteins including pp97 and pp45. At this latter concentration, tyrphostin also produced detachment of some MDCK cells from the plate which may have accounted for some of this inhibition in a non-specific manner.

In cells pretreated for 20 h with 200 μM RG 50864, EGF also failed to induce significantly *c-myc* and *c-fos* (Fig. 5). By comparison to EGF receptor autophosphorylation, however, this inhibition was virtually complete when phosphorylation was only partially blocked in the same cells using the same inhibitor concentration. This was not due to a non-specific effect of RG 50864 on mRNA synthesis, since under the same conditions, the level of polyA binding protein mRNA was not reduced.

Comparison of dose-response curves for EGF stimulated events in MDCK cells

Figure 6 compares the dose-response relationship between some of the early and late cellular events following EGF stimulation of MDCK cells. When compared to EGF receptor autophosphorylation, there was a significant increase in sensitivity to substrate (pp120) phosphorylation and DNA synthesis. By contrast, EGF induction of *c-fos* and *c-myc* mRNA required

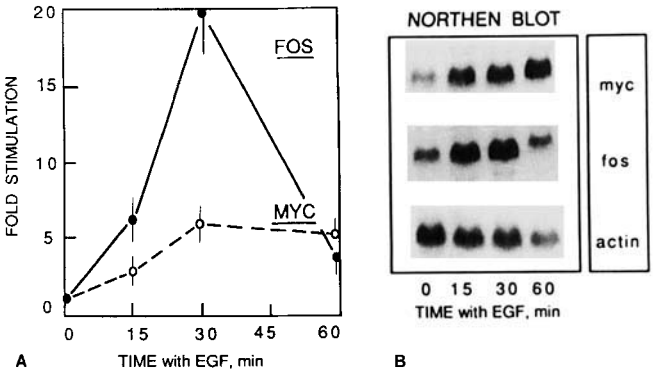

Fig. 3. Stimulation of *c-myc* and *c-fos* gene expression by EGF. Confluent MDCK cells were starved for 24 h in serum-free MEM and they were stimulated for 0 to 60 minutes with 10 nM EGF. RNA isolation was then performed using the low temperature 4 M guanidium thiocyanate-phenol-chloroform extraction technique followed by cold ethanol precipitation. Northern blot analysis was performed and the same blot was hybridized consecutively with *v-fos*, *c-myc*, and β-actin ^{32}P-DNA labeled probes. **A:** The signals specific for each mRNA species were quantified by scanning densitometry of the autoradiograms and the results were corrected for the signals obtained with β-actin whose gene expression is not stimulated by EGF. Data are expressed as fold stimulation over basal and each point represents the mean of 3 separate experiments. **B:** Representative Northern blot following EGF stimulation in intact cells. Ten micrograms of total cellular RNA were applied to each lane. Exposure time of autoradiograms was 24 h for proto-oncogenes and 48 h for β-actin.

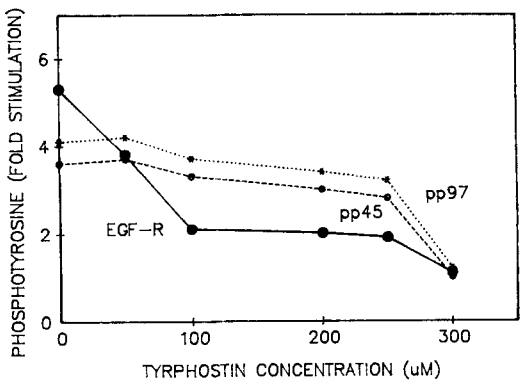

Fig. 4. Inhibition of EGF receptor protein kinase phosphorylation rate by RG 50864. MDCK cells were grown to confluence, serum-starved for 24 hours, and preincubated 20 h at 37°C in the presence of tyrphostin at the final concentrations indicated. After stimulation with 100 nM EGF for 10 minutes, cells were harvested and their phosphotyrosine-containing proteins were immunoprecipitated with antiphosphotyrosine antibody. The immunoprecipitates were analyzed by SDS-PAGE and immunoblotted with anti-phosphotyrosine antibody and ^{125}I protein A as described under Methods. The bands corresponding to the EGF receptor, and the EGF-induced 45 kD and 97 kD phosphoproteins were cut from the nitrocellulose filters and their radioactivity was determined in a gamma counter. Data are mean of two experiments.

significantly higher concentrations of EGF. The ED_{50} for oncogene expression 10–30 nM versus 3 nM for autophosphorylation, 1 nM for DNA synthesis, and <0.1 nM for pp120 phosphorylation. Thus, EGF pro-

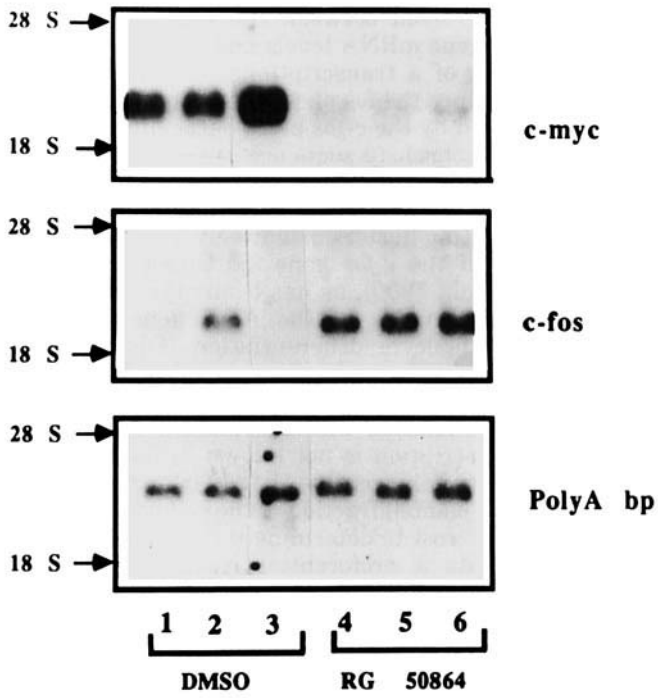

Fig. 5. Inhibition of EGF-stimulated proto-oncogene expression by tyrphostin (RG 50864). MDCK cells were grown to confluence and incubated for 2 days in serum-free MEM. The cells were then preincubated 20 hours at 37°C in the presence of 200 μM RG 50864 in DMSO or DMSO alone, prior to stimulation with 10 nM EGF for the indicated time intervals. RNA isolation was performed as described under Methods. Shown is a representative Northern blot. The same blot was first hybridized to *c-myc* and *c-fos* probes and ultimately to a cDNA coding for a polyA binding protein to control for the amount of RNA loaded onto each lane. Lanes 1, 2, 3, are 0, 30, 120 minutes, respectively, in the presence of EGF in the DMSO control cells. Lanes 4, 5, 6 are 0, 30, 120 minutes, respectively, in the presence of EGF in the "RG" treated cells.

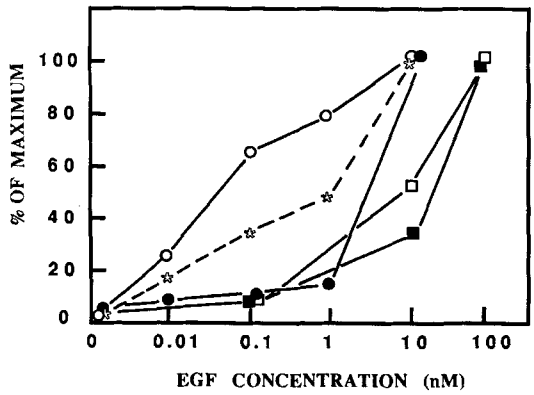

Fig. 6. Dose-response relationship of early and late cellular events elicited by EGF stimulation in intact MDCK cells. Shown are the phosphorylation of EGF receptor (●) and endogenous substrate pp120 (○) quantified by scanning densitometry of autoradiograms. Stimulation of *c-myc* (■) and *c-fos* (□) gene expression by EGF are evaluated after Northern blot analysis. The stimulation of DNA replication is evaluated by ^{3}[H]-thymidine incorporation into DNA (*). Serum starved cells were incubated with 10 nM EGF for 24 h and 2 μCi/ml ^{3}[H]-thymidine was added for 4 h; then TCA insoluble radioactivity was measured. The results for the stimulation of EGF-R, substrate phosphorylation, and proto-oncogene induction were analyzed by scanning densitometry. All results are expressed as percent stimulation over basal and represent the mean of 2–3 experiments.

duced about 50% of its maximal effect on cell growth at a concentration which had minimal effects on either receptor autophosphorylation or proto-oncogene induction.

DISCUSSION

As with a number of growth factors whose receptors belong to the receptor tyrosine kinase family, EGF interacts with specific membrane receptors to activate cell proliferation (reviewed in Yarden and Ullrich, 1988). The EGF receptor complex triggers a cascade of cellular responses that usually culminate in DNA synthesis and cell division (Sibley et al., 1987; Chen et al., 1987). In this study, we have examined the relationship between the expression of the cellular proto-oncogenes *c-myc* and *c-fos*, DNA synthesis, and EGF receptor tyrosine kinase activity in MDCK cells. We have demonstrated that in these cells, the intrinsic protein tyrosine kinase of the EGF receptor is highly stimulatable by EGF. A significant stimulation was detectable at 1 min and the half maximal increase in autophosphorylation was achieved 15 min after the onset of EGF stimulation (Fig. 2A,C). In these cells, EGF also had a delayed mitogenic effect with 10 nM EGF producing a doubling in the rate of DNA synthesis and a 40% increase in cell number. This ability of EGF to stimulate DNA synthesis in MDCK cells is comparable to that observed in other cell lines, such as NIH-3T3 fibroblasts (Aharonov et al., 1978; L'Allemain and Pouyssegur, 1986). In the carcinoma cell line A431 which has been mostly used to study EGF action, EGF actually inhibits cell growth (Macara, 1986). It has been suggested that EGF receptor tyrosine kinase activity is essential for stimulation of DNA synthesis, as exemplified by the inability of an in vitro mutant of the EGF receptor at the ATP binding site to induce this delayed response of EGF (Honegger et al., 1988).

In general, the rapid transcription of the proto-oncogenes *c-fos* and *c-myc* followed by the rapid accumulation of their mRNAs is among the earliest events implicated in gene expression following growth factor stimulation in a large number of cell lines (Muller, 1985; Muller et al., 1984; Forsthoefel and Thompson, 1987; Hall et al., 1987). The mitogenic potency of EGF has been related to its specific capacity to induce proto-oncogene expression (Cutry et al., 1989; Lindsten et al., 1988; Bravo et al., 1985). Similar findings have been found in cultured cells activated by platelet-derived growth factor (Armelin et al., 1984; Kelly et al., 1983) or insulin (Taub et al., 1987). Our results demonstrate that in intact MDCK cells, EGF regulates the expression of *c-fos* and *c-myc* genes in a time- and dose-dependent manner and that the rapid and transient induction of *c-fos* mRNA precedes *c-myc* gene activation as observed in fibroblasts (Muller et al., 1984). In addition, we have shown, using the EGF receptor tyrosine kinase inhibitor RG 50864, a compo-

nent belonging to the recently characterized family of tyrphostins (Lyall et al., 1989; Margolis et al., 1989), that the EGF stimulation of *c-myc* and *c-fos* mRNA levels could be totally abolished. This is consistent with previous experiments (Yaish et al., 1988), which have shown that at the concentration of tyrphostin used in these experiments (i.e., 200 μM) cell proliferation is totally suppressed. This occurs under conditions, however, in which receptor autophosphorylation has been only partially blocked. In addition, careful comparison of dose-response relationships reveal that induction of *c-fos* and *c-myc* is not part of a signal amplification process, but occurs only at EGF concentrations relatively higher than those required for stimulation of receptor autophosphorylation, substrate phosphorylation, and DNA synthesis stimulation.

These data, together with other data in the literature, suggest a more complex relationship between EGF's early and late events at the cellular level. Clearly, evidence from multiple points of view, but especially from in vitro mutagenesis, suggests that receptor tyrosine kinase activity is required for signal transduction by the EGF receptor (Honegger et al., 1987). Receptors which lack ATP binding and kinase activity fail to produce any stimulation of substrate phosphorylation, induce proto-oncogenes, or stimulate DNA synthesis. Likewise, inhibitors of the EGF receptor kinase, such as the tyrphostins, have been shown to block EGF actions in 3T3 cells (Lyall et al., 1989). In the present study, the discordances are quantitative rather than qualitative. EGF stimulates DNA synthesis at concentrations below those required for maximal receptor autophosphorylation consistent with spare receptors and signal amplification. At least one component of this amplification appears to be substrate phosphorylation as viewed by pp120 phosphorylation. Induction of *c-myc* and *c-fos*, on the other hand, occurs only at high levels of receptor activation and does not represent a step in signal amplification. Furthermore, the modest inhibition of EGF receptor kinase activity produced by tyrphostin suggests that other kinases or other post-receptor steps involved in the stimulation of oncogene induction have been blocked by this class of agents. In this respect it has been shown that the transcription of the *c-fos* gene can be regulated by cAMP dependent protein kinase and/or protein kinase C depending on the growth factor and the cell line considered (Bravo et al., 1987; Gilman, 1988). Although it has been shown that tyrphostins inhibit these kinases only partially in vitro, even at millimolar concentrations (Yaish et al., 1988), one cannot rule out that in intact cells the micromolar concentrations of RG 50864 that were used in this study could have decreased the activity of these or other kinases. In addition, the activation of the *c-myc* and *c-fos* proto-oncogenes may require the generation of other second messengers such as those generated through regulation of the inositol lipid cycle (Pike and Eakes, 1987; Doglio et al., 1989). In A431 cells, RG 50864 has been shown to block EGF induced tyrosine phosphorylation of phospholipase C II, another potential endogenous substrate of the receptor kinase and possible mediator of *c-myc* and *c-fos* induction pathways (Margolis, et al., 1989).

These results leave open the way to elucidate the direct steps involved between the rapid cellular increase in oncogene mRNA levels and cell proliferation. The possibility of a transcriptional control has been hypothesized since the fos phosphoprotein (i.e., the end product encoded by the *c-fos* proto-oncogene) is known to bind a DNA consensus sequence in a number of genes through protein-protein interactions (Rauscher et al., 1988; Turner and Tjian, 1989). Such protein complexes act as transacting factors negatively regulating the transcription of the *c-fos* gene (Sassone-Corsi et al., 1988; Ofir et al., 1990), as has been observed for the transciptional complex of the AP-2 gene promotor involved in adipocyte differentiation (Distel et al., 1987). This mechanism of action may also be effective in promoting long-term cellular responses by altering other gene expression, but the precise signal that regulates transcription is not known so far. Since, at any given time, it is possible that EGF induces mitogenesis in only a small fraction of the cell population, it would be of interest to determine if this same fraction of cells exhibits a preferential induction of proto-oncogene expression and/or tyrosine kinase activation.

In conclusion, we have shown that EGF is capable of stimulating early (*c-myc* and *c-fos* oncogene induction) and delayed (DNA synthesis) biological effects in intact MDCK cells. The magnitude of the EGF-induced responses is not directly related to the stimulation of EGF receptor tyrosine kinase activity. These results strongly suggest that an alternate mechanism, such as protein kinase C or phospholipase C activation, contributes to the pleiotropic cellular effects characteristic of EGF.

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