Carboxy-terminal truncations of epidermal growth factor (EGF) receptor affect diverse EGF-induced cellular responses

W. Li,* N. Hack,† B. Margolis,* A. Ullrich,‡ K. Skorecki,† and J. Schlessinger*  
*New York University Medical Center  
Department of Pharmacology  
New York, New York 10016  
†MRC Group in Membrane Biology  
Division of Nephrology  
Toronto General Hospital  
Toronto, Canada M5G2C4  
‡Max Planck Institut für Biochemie  
8033 Martinsreid  
Germany

The binding of epidermal growth factor (EGF) to its receptor induces tyrosine phosphorylation of phospholipase Cγ (PLCγ), which appears to be necessary for its activation leading to phosphatidylinositol (PI) hydrolysis. Moreover, EGF-receptor (EGF-R) activation and autophosphorylation results in binding of PLCγ to the tyrosine phosphorylated carboxy-terminus of the receptor. To gain further insights into the mechanisms and interactions regulating these processes, we have analyzed transfected NIH-3T3 cells expressing two EGF-R carboxy-terminal deletion mutants (CD63 and CD126) with reduced capacity to stimulate PI hydrolysis, Ca2+ rises, and DNA synthesis. In fact, the CD126 mutant lacking 126 carboxy-terminal amino acids, including four tyrosine autophosphorylation sites, was unable to stimulate PI hydrolysis or Ca2+ rise in response to EGF. Surprisingly, EGF binding to the cell lines expressing CD63 or CD126 mutants was followed by similar stimulation of tyrosine phosphorylation of PLCγ. Our results suggest that although necessary, tyrosine phosphorylation of PLCγ may not be sufficient for stimulation and PI hydrolysis. It is clear, however, that the carboxy-terminal region of EGF-R is involved in regulation of interactions with cellular targets and therefore plays a crucial role in postreceptor signaling pathways.

Introduction

Serum growth factors stimulate cell growth and differentiation by binding to and activating cell surface receptors that possess intrinsic, ligand-sensitive, protein tyrosine kinase activities. These surface receptors contain multiple functional domains including extracellular ligand-binding domain, transmembrane spanning region, catalytic tyrosine kinase domain, and flanking regularity sequences (reviewed by Ullrich and Schlessinger, 1990). It has been recently appreciated that specific sequences containing tyrosine phosphorylation sites of growth factor receptors are binding sites for target proteins containing a sequence motif termed SH2 (src homology-2) (reviewed by Pawson, 1988). These include PP60c-src, phospholipase Cγ (PLCγ), GTPase activating protein (GAP), v-crk, and the phosphatidylinositol (PI)-3 kinase associated P85 protein (Anderson et al., 1990; Margolis et al., 1990a; Moran et al., 1990; Skolnik et al., 1991).

The carboxy-terminal regions are among the most divergent sequences of all known receptor tyrosine kinases and also contain multiple tyrosine autophosphorylation sites (reviewed in Ullrich and Schlessinger, 1990). The major tyrosine autophosphorylation sites of epidermal growth factor (EGF) receptor (EGF-R) are clustered in the carboxy-terminal tail at positions Y1086 (Hsuan et al., 1989; Margolis et al., 1989a), Y1068, Y1148, and Y1173 (Downward et al., 1985). Although the regulatory role of autophosphorylation has been demonstrated in some tyrosine kinases, for example PP60c-src (Kmiecik and Shalloway, 1987; Piwnica-Worms et al., 1987) or insulin receptor (Rosen et al., 1983), no profound and consistent role of EGF-R autophosphorylation has been established. Single mutations, from tyrosine to phenylalanine, of individual tyrosine autophosphorylation sites at positions Y1068, Y1148, or Y1173 did not have a major effect on EGF-R-mediated cellular responses (Honegger et al., 1988a,b). It has been reported that truncation of 34 carboxy-terminal amino acids, containing Y1173, enhanced cell transformation (Khazaie et al., 1988). However, larger truncation of 126 amino acids containing all four tyrosine autophosphorylation sites abolished transforming ca-
capacity in erythroblasts but not in fibroblasts (Khazaie et al., 1988). It was reported that larger carboxy-terminal truncations from residue 1052 to 957 resulted in enhanced protein tyrosine phosphorylation in NIH-3T3 cells (Chen et al., 1989), suggesting that the carboxy-terminus plays an inhibitory role in the regulation of receptor tyrosine kinase activity. However, mutant receptors with shorter deletion of 19, 63, or 64 amino acid residues were less efficient than wild-type EGF-R in induction of EGF-dependent transformation and anchorage-independent growth of either 3T3 or RAT1 cells (Haley et al., 1988; Velu et al., 1989).

In this report, we compare EGF-induced cellular responses of two deletion EGF-R mutants with EGF-induced cellular responses of wildtype EGF-R expressed in NIH-3T3 cells. It appears that a 126 amino acid deletion at carboxy-terminal tail of EGF-R prevents EGF-induced phosphatidylinositol turnover and Ca\textsuperscript{2+} signaling. However, only a marginal change was observed for EGF-induced tyrosine phosphorylation of PLC\textgamma in these cells. Our data support the notion that the carboxy-terminal tail of EGF-R has a differential regulatory role on various signal transduction pathways. Moreover, it reveals the complexity of receptor-substrate interactions and regulation.

Results

Carboxy-terminal deletion of EGF-R abolishes PI turnover and Ca\textsuperscript{2+} rise

The binding of EGF to EGF-R causes a transient increase in cytoplasmic Ca\textsuperscript{2+}. To explore the mechanisms underlying Ca\textsuperscript{2+} release we have compared the capacity of two previously described deletion mutants of EGF-R (Livneh et al., 1986; Khazaie et al., 1988; Margolis et al., 1989a) lacking 63 (CD63) or 126 (CD126) amino acids from the carboxy-terminal tail, respectively, to stimulate Ca\textsuperscript{2+} release from intracellular compartments. EGF-induced Ca\textsuperscript{2+} release was measured by using the Ca\textsuperscript{2+} indicator indo-1 as previously described (Margolis et al., 1990b; Hack et al., 1991). Figure 1 shows that EGF-induced Ca\textsuperscript{2+} rise was significantly reduced in cells expressing CD63 deletion mutant and completely abolished in cells expressing the CD126 deletion mutant. Similar results were obtained when three different cell lines expressing varying amounts of CD126 deletion mutants were compared. These cell lines express 1.5 \times 10^6 receptors/cell (CD126, Figure 1), 1 \times 10^6 receptors/cell (CD126/D2), or 1.2 \times 10^6 receptors/cell (CD126/D4) (data not shown).

The capacity to stimulate phosphatidylinositol-4,5-phosphate (PIP\textsubscript{2}) breakdown by phospholipase C in response to EGF was also reduced in cells expressing the deletion mutants, CD63 and CD126 (Figure 2). In this experiment

![Figure 1. Effect of carboxy-terminal truncations of EGF-R on EGF-induced Ca\textsuperscript{2+} rise. HER14, CD63, and CD126 cells suspended in PBS were incubated with Indo-1-ace- toxymethyl ester (2.5 \muM) for 30 min at 37°C, washed free of extracellular dye, and incubated with or without 40 nM EGF. Fluorescence measurements at the peaks and calibration to free cytosolic Ca\textsuperscript{2+} concentration were performed as previously described (Margolis et al., 1990b). A representative of 6 independent reproducible experiments is presented. Results are the means ± SEM (triplicate data points).](image1)

![Figure 2. Effect of carboxy-terminal truncations of EGF-R on EGF-induced phosphatidylinositol turnover. Cells labeled with [\textsuperscript{3}H]myo-inositol (see Materials and methods) were incubated in the presence or absence of 40 nM EGF for 30 min at 37°C. The inositol phosphates were extracted and applied to Bio-Rad A6G × 8 columns. The total inositol phosphates were eluted and counted in a Scintillation counter. Results are means ± SEM in fold increases of nine independent experiments.](image2)
the cells were labeled with [3H]myoinositol and then incubated in the presence or absence of EGF for 30 min at 37°C as previously described (Margolis et al., 1990b). It can be seen that the addition of EGF caused phospholipase C-mediated increase in PI turnover in HER14 cells and to a lower extent in CD63 cells. However, no EGF-induced PI turnover was detected in CD126 cells. Similarly, EGF-induced PI breakdown was not detected in CD126/D2 and CD126/D4, expressing high amounts of CD126 mutant (Figure 2 and data not shown). Hence, deletion of 126 carboxy-terminal amino acid residues containing four tyrosine autophosphorylation sites abolishes both EGF-induced PI turnover and Ca2+ release.

**Tyrosine phosphorylation of PLCγ in cells expressing wt or deletion mutants of EGF-R**

We have shown recently that the tyrosine phosphorylated carboxy-terminal tail of EGF-R is a major site for association with various SH2-containing proteins such as PLCγ1, GAP, and PI3 kinase-associated P85 (Margolis et al., 1990a; Skolnik et al., 1991). Moreover, growth factor-induced tyrosine phosphorylation of PLCγ was shown to be essential for activation of phospholipase C activity (Nishibe et al., 1990; Kim et al., 1991). With this in mind, together with the fact that EGF is unable to enhance PI turnover and Ca2+ release in CD126 cells, we expected that tyrosine phosphorylation of PLCγ be reduced in CD63 and perhaps even abolished in cells expressing CD126 mutant. Surprisingly, EGF-induced tyrosine phosphorylation of PLCγ was detected in HER14, CD63, and CD126 cells (Figure 3). In these experiments the various cell lines were [32P] labeled and stimulated with EGF for 2 min at room temperature. After solubilization, the phosphotyrosine (PY) containing proteins were separated by immobilized anti-PY antibodies (see Materials and methods section) and submitted to immunoprecipitation analysis with either anti-EGF-R (lanes 1, 3, 5) or anti-PLCγ antisera (lanes 2, 4, 6), followed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and autoradiography. Figure 3A shows that tyrosine phosphorylated PLCγ was detected in EGF-treated HER14 (lane 2), CD63 (lane 4), and CD126 (lane 6) cells. Clearly, EGF is able to stimulate tyrosine phosphorylation of PLCγ not only in HER14 but also in CD63 and CD126 cells. For better quantification of tyrosine-phosphorylated PLCγ, the different cell lines (HER14, CD63, or CD126) were first metabolically labeled with [35S]methionine, treated with EGF, lysed, and immunopurified with anti-PY antibodies. The PY fractions were then subjected to analysis by immunoprecipitation with either anti-PLCγ or anti-EGF-R antibodies. Again this experiment shows that deletions in carboxy-terminal tail of the EGF-R have a marginal effect on the capacity of EGF to induce tyrosine phosphorylation of PLCγ (Figure 3B). Quantitation of tyrosine phosphorylation of PLCγ induced by EGF was determined by Scintillation measurements of radioactive contents of anti-PY immunoprecipitated PLCγ bands or by densitometric analysis. Approximately twofold higher phosphate incorporation of PLCγ was found in CD63 cells after normalization for equal receptor numbers (Figure 3A). However, similar amounts of tyrosine phosphorylated PLCγ was found in HER14, CD63, and CD126 cells labeled with [35S]methionine and immunoprecipitated with PY antibodies (Figure 3B). In all, EGF-induced tyrosine phosphorylation of PLCγ1 does not correlate with PI turnover and Ca2+ rise induced by EGF in cells expressing deletion mutants CD63 and CD126.

**EGF-induced tyrosine phosphorylation of cellular proteins in HER14, CD63, or CD126 cells**

We then compared the total EGF-induced protein tyrosine phosphorylation in these cells. Cells labeled with [32P]orthophosphate were incubated in the presence or absence of 40 nM EGF at 25°C for 2 min; conditions were shown to be optimal for maximum recovery of tyrosine phosphorylation in these cells (Figure 4A). Similar kinetics of tyrosine phosphorylation were observed in HER14, CD63, and CD126 cells (data not shown). The PY-containing proteins were isolated by anti-PY affinity chromatography as described (see Materials and methods) and subsequently analyzed by 5–15% (acylamide) gradient SDS gel and by autoradiography (Figure 4B). A similar pattern of EGF-induced tyrosine phosphorylated proteins was observed in HER14 (lanes 1, 2), CD63 (lanes 3, 4), and CD126 (lanes 5, 6) cells. However, minor but significant differences in tyrosine phosphorylation were observed in the different cell lines. A 124-kDa protein was tyrosine phosphorylated in response to EGF in HER14 but not in CD63 or CD126 cells. Similarly a 130-kDa protein was found to be tyrosine phosphorylated in CD63 and CD126 cells but not in HER14 cells. Finally, an EGF-stimulated
Figure 3. EGF-induced tyrosine autophosphorylation of EGF-R and tyrosine phosphorylation of PLC-γ. (A) [32P]Orthophosphate- or (B) [35S]methionine-labeled cells were incubated in the presence (+) or absence (−) of 40 nM EGF for 2 min at 25°C. Cells were solubilized and the anti-phosphotyrosine (αPY)-reactive proteins were isolated (see Materials and methods). The αPY-reactive fractions were immunoprecipitated with either anti-EGF-R antibodies (lanes 1, 3, 5) or anti-PLC-γ antiserum (lanes 2, 4, 6) and analyzed by SDS-PAGE and autoradiography. The scintillation counts (cpm) of the excised PLC-γ bands from [35S]-labeled HER14, CD63, and CD126 cells are 868, 685, and 763, respectively. Exposure times: (A) 48 h; (B) 4 d.

42-kDa tyrosine phosphorylated protein was present in HER14 (lane 2) but absent in CD63 (lane 4) or CD126 (lane 6) cells, and instead an EGF-stimulated 44-kDa protein was found to be phosphorylated only in CD63 and CD126 cells. The difference in intensity of tyrosine phosphorylation of cellular proteins of the various cell lines was in accord with the level of surface expression of wt and the two mutant receptors. Direct binding experiments with [125I]-EGF and Scatchard analysis indicated that HER14 cells express $4 \times 10^5$ receptors/cell, whereas CD63 and CD126 express $4.6 \times 10^5$ and $1.5 \times 10^5$ receptors/cell, respectively. Our data show that deletions in the carboxy-terminal tail of EGF-R do not have a profound effect on the pattern of tyrosine phosphorylation. Nevertheless, it is possible that the minor differences in the pattern of tyrosine phosphorylation observed in CD63 and CD126 cells have a significant impact on the activation of various cellular signaling pathways.
Figure 4. EGF-stimulated protein tyrosine phosphorylation in intact HER14, CD63, or CD126 cells. [32P]orthophosphate-labeled cells were incubated with (+) or without (−) 40 nM EGF at 25°C. Cells were solubilized and the αPY-reactive proteins isolated (see Materials and methods) and subjected to SDS-PAGE and autoradiography. (A) Kinetics of EGF-stimulated protein tyrosine phosphorylation in HER14 cells. (B) Comparison of EGF-stimulated (after 2 min) protein tyrosine phosphorylation in HER14, CD63, and CD126 cells. Exposure time: (A) 8 h; (B) 6 h.

Carboxy-terminal deletions impair EGF-induced mitogenesis

We have compared the capacity of EGF to stimulate DNA synthesis and proliferation of HER14, CD63, and CD126 cells. The various cell lines were starved in serum-free medium for 24 or 48 h and then incubated with different concentrations of EGF for 18 h before the addition of [3H]thymidine. In addition, we have compared EGF-induced growth curves of HER14, CD63, and CD126 cells. The results of both methods were consistent. Moreover, the capacity of EGF to induce thymidine incorporation in the two cell lines that overexpress deletion mutant CD126, namely CD126/D2 (1 × 10⁸ receptors/cell) and CD129/D4 cells (1.2 × 10⁸ receptors/cell), was also compared. In spite of the fact that in different experiments significant variation was ob-
served for the thymidine incorporation results of the various cell lines, the overall trend indicated the CD63 and CD126 were less responsive to EGF than HER14 cells. However, HER14, CD63, and CD126 were similarly responsive to 10% fetal calf serum. Figure 5, A and B shows that the ability of EGF to induce thymidine incorporation and cell proliferation is severely reduced in CD63 and CD126 cells.

Discussion

The carboxy-terminal tails of growth factor receptors with tyrosine kinase activity are thought to function as regulatory regions. Hence, several reports describe the analysis of EGF-R mutants with altered carboxy-terminal tails. These include point mutations (Bertics et al., 1988; Honegger et al., 1988a,b) or various deletion mutants (Haley et al., 1988; Khazaie et al., 1988; Velu et al., 1989). Nevertheless, the conclusions drawn from these analyses are inconsistent and confusing. Whereas certain studies suggest that the carboxy-terminal tail has an autoinhibitory function (Bertics et al., 1988; Honegger et al., 1988a,b; Chen et al., 1989) other studies support the view that this region has a stimulatory role in cell growth and transformation (Downward et al., 1984).

Recent studies indicate that growth factors such as platelet-derived growth factor, EGF or fibroblast growth factor are able to stimulate tyrosine phosphorylation of PLC-γ (Margolis et al., 1989a,b; Meisenhelder et al., 1989; Wahl et al., 1989; Margolis et al., 1990) and that tyrosine phosphorylation activates the enzyme (Margolis et al., 1990a; Nishibe et al., 1990; Kim et al., 1991) leading to enhanced PIP_2 hydrolysis. We have recently shown that the tyrosine phosphorylated carboxy-terminal tail of EGF-R is a major site for association with PLCγ and other SH2-containing proteins such as GAP and PI3 kinase associated P85 (Margolis et al., 1990a; Skolnik et al., 1991). Consistent with this view is the result shown in this report that EGF failed to induce PIP_2 turnover and Ca^{2+} release in cells expressing CD126; a mutant receptor lacking 126 carboxy-terminal residues including four tyrosine autophosphorylation sites. We have interpreted these results as an indication that deletion of the carboxy-terminal region prevented association with and tyrosine phosphorylation of PLCγ, which therefore could not be activated, thus preventing EGF-induced PIP_2 hydrolysis. This simple explanation is apparently incorrect as we show in this report that EGF is able to stimulate tyrosine phosphorylation of

![Graph](image)

**Figure 5.** EGF-induced mitogenic responses in the wild-type and mutant cell lines. (A) EGF-induced thymidine incorporation. Subconfluent cells were starved in serum-free medium and then incubated with different concentrations of EGF before the addition of [H]thymidine (see Materials and methods). This experiment was repeated three times with similar results. HER14 (○), CD63 (△), CD126 (□), CD126/D2 (+), and CD126/D4 (■-■). In control experiments 10% fetal calf serum had 10- to 40-fold stimulation of thymidine incorporation in the various cell lines. (B) EGF-induced growth curves. Serum-starved cells were incubated without (open symbols) or with (solid symbols) either 10% fetal
Signaling by EGF-receptor mutants

PLCγ, in HER14, as well as in CD63 and CD126 cells. Clearly, EGF-induced tyrosine phosphorylation of PLCγ does not correlate with EGF-induced PI hydrolysis and Ca²⁺ release in these cells. The reason for this discrepancy is not clear. Several mechanisms can be put forward to explain this paradoxical result.

The carboxy-terminal tail of EGF-R contains at least four tyrosine autophosphorylation sites and therefore this region may compete with exogenous substrates for substrate binding site on the catalytic kinase domain. The carboxy-terminal region, according to this model, provides an autoinhibitory role (reviewed by Ullrich and Schlessinger, 1990). However, substrates containing SH2 domains, such as PLCγ, bind avidly to the tyrosine phosphorylated tail, increasing the affinity of PLCγ toward the activated EGF-R and therefore decreasing the Km for tyrosine phosphorylation of PLCγ. The carboxy-terminal tail of EGF-R has opposing functions: on one hand it directs interactions with SH2 containing target substrates and on the other hand it provides an autoinhibitory function for substrate phosphorylation. The balance between these two functions could play an important role in the physiological regulation of substrate recognition and phosphorylation. Truncation of the carboxy-terminal tail of EGF-R will remove both constraints and therefore as a net result the extent of tyrosine phosphorylation of PLCγ may be marginally influenced in CD126 as compared with HER14 cells.

Recent evidence provide support for the view that growth factor-induced tyrosine phosphorylation of PLCγ is essential for its activation leading (Nishibe et al., 1990; Kim et al., 1991) to PIP₂ hydrolysis. Moreover, there is evidence that tyrosine phosphorylation does not directly enhance phospholipase C activity but rather releases an inhibitory constraint (Kim et al., 1991). The nature of this inhibitory factor is not established nor is it clear whether it is tyrosine phosphorylated or able to interact with the carboxy-terminal tail of EGF-R.

In addition, it is possible that PLCγ is able to interact with EGF-R through unknown linker proteins that bind to region(s) on the EGF-R molecule other than the carboxy-terminal tail. An additional possibility is that the association between PLCγ and EGF-R and tyrosine phosphorylation of PLCγ by EGF-R kinase are two unrelated phenomena. However, this explanation is unlikely because our preliminary results showed that the presence of SH2 domain decreases the Km for tyrosine phosphorylation for PLCγ by the EGF-R kinase (Rotin et al., unpublished data). Yet, it is clear that deletion in the carboxy-terminal tail have minor qualitative effect on the pattern of tyrosine phosphorylation of cellular substrates. The tyrosine phosphorylation of these as yet unknown substrates may play a role in regulation of different signaling pathways including PI hydrolysis and Ca²⁺ release.

Our results show that deletion of 63 amino acids from the carboxy-terminal tail has a profound effect on Ca²⁺ release. This result in conflict with Chen et al. (1989), who reported that an amino acid segment between residues 973 and 991 is critical for Ca²⁺ signaling. Our results are inconsistent with the notion that EGF-R has a specific Ca²⁺ regulatory domain. Moreover, we did not detect a major increase in the total tyrosine phosphorylation of total cellular proteins in cells expressing carboxy-terminal deletion mutants (Chen et al., 1989). The reason for these discrepancies are not known.

It is clear, however, that the carboxy-terminal tail has a profound effect on EGF-induced DNA synthesis. Yet, on the basis of our current knowledge it is impossible to correlate between the effects of carboxy-terminal deletion on early events and the reduced mitogenic activity. Nevertheless, this analysis provides further support for the central, yet not fully understood, role of the carboxy-terminal tail in regulation of receptor action.

Material and methods

NIH-3T3 cells expressing wt human EGF receptor (HER14) or carboxy-terminal truncation mutants (CD63 and CD126) were previously described (Margolis et al., 1989). Mouse EGF was purchased from Toyoobo Co. (Osaka, Japan). [¹²⁵I]myo-inositol (carrier-free, 3.7 GBq/ml) and [³²P]myo-inositol (2.94–4.44 TBq/mmol) was from Amersham Corporation (Arlington Heights, IL). [³²P]Orthophosphate (carrier-free, >300 TBq/mmol) and tritiated thymidine, specific activity 6.7 Ci/mmol were from New England Nuclear (Wilmington, DE). [³⁵S]Methionine (>1000 Ci/mmol) was purchased from ICN Biochemicals, Inc. (Irvine, CA). Indo-1 was from Molecular Probes, Inc. (Eugene, OR). Monoclonal anti-phosphotyrosine (aPY) antibody (1G2) covalently coupled to sepharose 4B was from Oncogene Science, Inc. (Manhasset, NY). Monoclonal anti-EGF receptor antibody (mAb 108) and anti-PLCγ antisera were used as described (Margolis et al., 1989; Bellot et al., 1990). Other reagents, unless indicated, were purchased from Sigma (St. Louis, MO).

calf serum (upper right) or 1% fetal calf serum plus 5 nM EGF for the indicated times. Cell numbers were determined by counting in a Coulter Counter. Results are means of duplicate dishes of two independent experiments. HER14 (○, ●); CD63 (△, ◆); CD126 (□, ■).
Cell labeling and analysis of protein tyrosine phosphorylation

For 32P-orthophosphate labeling, cells cultured in Dulbecco’s modified Eagle’s medium with 10% calf serum in 60-mm tissue culture dishes to subconfluence were starved in the medium containing 0.5% calf serum for 16 h. The cells were washed three times with phosphate-free medium (Eagle’s minimum essential medium [MEM]; Gibco, Grand Island, NY) and incubated for a further 1 h at 37°C in the wash medium supplemented with 0.5% dialyzed calf serum. The cells were washed three times with the phosphate-free medium again before the incubation for 2 h at 37°C in the same medium containing 0.5% dialyzed calf serum and [32P]orthophosphate (1 μCi/ml), pH 7.2. For [32P]methionine labeling, cells in 100-mm tissue culture dishes were washed three times in methionine-free medium (MEM Deficient, Sigma) and incubated for 15 h in the same medium supplemented with 0.5% dialyzed calf serum, 5 μM methionine, and 150 μCi/ml [32P]methionine.

Radiolabeled cells were incubated in the presence or absence of mouse EGF (40 nM) for the indicated times. Incubations were stopped by rapidly aspirating the medium and washing the cells two times with ice-cold phosphate-buffered saline (PBS). Cells were solubilized by addition of ice-cold solubilization buffer (10 mM tris/hydroxymethylaminomethane-base, 50 mM NaCl, 5 mM sodium pyrophosphate, 50 mM sodium fluoride, 100 μM sodium orthovanadate, 2 mM iodoacetic acid, 1 mM phenyl methyl sulfonyl fluoride, 5 μM ZnCl2, 1% Triton X-100, pH 7.1) (1 ml per 60-mm dish; 1.5 ml per 100-mm dish). Insoluble material was removed from lysates by centrifugation (14,000 rpm, 25 min, 4°C in Eppendorf Centrifuge, Brinkmann Instruments, Germany). Protein concentrations were determined by the method of Bradford (1976), and the total protein content of each lysate matched before immunoprecipitation. Isolation of phosphotyrosyl proteins by αPY antibody affinity chromatography was done as described (Li et al., 1991). The αPY-reactive EGF-Rs and PLCγ were isolated by a secondary immunoprecipitation of the αPY-reactive fractions with either anti-EGF-R antibody or anti-PLCγ antisera. The total αPY-reactive proteins or the secondary immunoprecipitates were boiled for 5 min in electrophoresis sample buffer in the presence of 0.1 M β-mercaptoethanol and subjected to gradient (7–15% acrylamide) SDS-PAGE at constant voltage (120 V) at 15°C. Gel containing 32P-labeled samples were impregnated with Amplify (Amersham) for 30 min in the dark before drying and autoradiography.

Analyses of EGF-stimulated changes in inositol phosphate generation and the intracellular Ca2+ concentration

The sum of all inositol phosphates and the rise in intracellular Ca2+ concentrations were measured as described previously (Margolis et al., 1990b; Hack et al., 1991).

EGF-induced thymidine incorporation and cell growth

Cells were seeded in 24-well tissue culture dishes and grown to subconfluence (4 × 104 cells/well). Cells were then incubated at 37°C in the serum-free medium for 36 h before incubation in the presence or absence of calf serum (10%) or indicated concentrations of mouse EGF for 18 h. [3H]Thymidine (1 μCi/ml) was added and further incubated for 4 h at 37°C. The incorporation of radioactivity into acid-insoluble material was measured as described (Margolis et al., 1990b). For measuring cell growth, serum-starved cells (~5× 104 cells/well) were incubated in the presence or absence of 5 nM EGF in the media containing either 1% or 10% calf serum for the indicated days. Media were removed and cells were washed twice with PBS and trypsinized. The cell numbers were determined in a Coulter counter.

Acknowledgments

We thank A. Sorokin for preparation of 125I-labeled EGF. This work is supported by a grant from RPR Central Research (U.S.), by a grant from Human Frontier Science Program (HFSP) (U.S.), and by a grant from Lucille P. Markey Trust (B.M.).

Received: May 2, 1991.
Revised and accepted: June 13, 1991.

References


