Transmembrane signalling at the epidermal growth factor receptor

Positive regulation by the C-terminal phosphotyrosine residues

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Mutant epidermal growth factor (EGF) receptors (obtained by substitution of one, two or three C-terminal autophosphorylatable tyrosine residues with phenylalanine residues or by deletion of the C-terminal 19 amino acids, including the distal tyrosine) were expressed in mouse NIH-3T3 fibroblast clones at densities comparable (< 25% difference) with those in control clones expressing the wild-type receptor. Total EGF-induced phosphorylation of the mutated receptors was not appreciably changed with respect to controls, whereas autophosphorylation at tyrosine residues was decreased, especially in the double and the triple mutants. In the latter mutant, expression of the EGF-receptor-activated lipolytic enzyme phospholipase C γ was unchanged, whereas its tyrosine phosphorylation induced by the growth factor was lowered to approx. 25% of that in the controls. In all of the cell clones employed, the accumulation of inositol phosphates induced by treatment with fetal calf serum varied only slightly, whereas the same effect induced by EGF was consistently lowered in those lines expressing mutated receptors. This decrease was moderate for those receptors missing only the distal tyrosine (point and deletion mutants), intermediate in the dual mutants and almost complete in the triple mutants. Likewise, increases in intracellular Ca²⁺ concentrations ([Ca²⁺]_i) induced by fibroblast growth factor were approximately the same in all of the clones, whereas those induced by EGF were decreased in the mutants, again in proportion to the loss of the phosphorylatable C-terminal tyrosine residues. The same trend occurred with membrane hyperpolarization, an effect secondary to the increase in [Ca²⁺]_i via the activation of Ca²⁺-dependent K⁺ channels. We conclude that C-terminal autophosphorylatable tyrosine residues play a positive role in the regulation of transmembrane signalling at the EGF receptor. The stepwise decrease in signal generation observed in single, double and triple point mutants suggest that the role of phosphotyrosine residues is not in the participation in specific amino acid sequences, but rather in the introduction of strong negative charges at strategic sites of the receptor tail. As a consequence of autophosphorylation, the receptor could become competent for specific association with phospholipase C γ , with ensuing activation by tyrosine phosphorylation followed by the chains of intracellular responses ultimately leading to DNA synthesis and cell duplication.

INTRODUCTION

Extensive evidence demonstrates that, analogous to other growth factors, epidermal growth factor (EGF) has its effects via the activation of tyrosine kinase activity in the cytoplasmic domain of its receptor (EGFR) and the phosphorylation of specific intracellular substrates (for reviews see [1–4]). A few of these substrates have been identified, and some are recognized to be enzymes or enzyme regulators of fundamental importance in cellular control [5–8]. Tyrosine phosphorylation is believed to modify the functioning of these molecules, thus initiating chain(s) of events which, in many cell types, ultimately lead to DNA replication and cell division. Among EGF substrates, one which has attracted a great deal of attention during the past few years is phospholipase C γ (PLC γ), an enzyme mostly located in the soluble cytosol which, via the hydrolysis of phosphatidylinositol 4,5-bisphosphate, induces the generation of two well-known second messengers, inositol 1,4,5-trisphosphate and diacylglycerol [9–13]. The former messenger is known to activate release of Ca²⁺ from intracellular rapidly-exchanging Ca²⁺ stores; the second activates protein kinase C [14] and, possibly, other important intracellular regulators [15]. Previous studies have already demonstrated that, in intact cells, a fraction of the PLC γ molecules is physically bound to the EGFR to form a complex

[9–13], and that this complex is no longer formed when mutated, tyrosine kinase-incompetent, receptors are expressed. The phosphorylations necessary for receptor–enzyme association have recently been shown to reside in the EGFR C-terminal tail, i.e. to result from the process of autophosphorylation at residues 1173, 1148 and 1068 [16,17]. Whether EGFR autophosphorylation is important for PLC γ activation is, however, still unclear. In fact, when mutants of EGFR with their whole C-terminal tail deleted were studied [18,19], the increase in the free cytosolic Ca²⁺ concentration [Ca²⁺]_i induced by the growth factors (i.e. a consequence of PLC γ activation) was found not to be inhibited, but rather to be slightly increased, provided that a short amino acid sequence (residues 973–991, more upstream of the autophosphorylation sites) was kept intact [19]. Large deletions, such as those investigated in the above studies, could however modify considerably the structure of the EGFR, and thus overwhelm unspecifically an autophosphorylation-relieved inhibitory regulation. In order to investigate this problem, we have now chosen to employ more subtle EGFR mutants, i.e. receptors either point-mutated in the three tyrosine residues or missing only the last few amino acids [20,21]. These mutants were expressed in NIH-3T3 fibroblasts at levels comparable with those in the controls, which express wild-type receptors. The results obtained by measuring phosphorylation of EGFR and PLC γ in

Abbreviations used: EGF, epidermal growth factor; EGFR, EGF receptor; [Ca²⁺]_i, cytosolic concentration of free ionized calcium; InsP₃, inositol phosphate; KRH, modified Krebs–Ringer medium buffered with Hepes; DMEM, Dulbecco's modified Eagle's medium; FGF, fibroblast growth factor; PLC γ , phospholipase C γ ; FCS, fetal calf serum.

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parallel to phosphoinositide hydrolysis, $[Ca^{2+}]_i$, and membrane potential demonstrate that C-terminal tyrosine phosphorylation is very important for the receptor interaction with, and activation of, the lipolytic enzyme, and possibly with other substrates as well.

MATERIALS AND METHODS

Transfection and cell culture

Construction of the various EGFR mutants and characterization of the NIH-3T3 clones are described in detail elsewhere [20,21]. A deletion mutant (Dc19) was obtained by removing the last 19 amino acids from the full-length EGFR. This mutant therefore does not contain the final tyrosine residue [20]. In some experiments a deletion mutant missing the distal 63 residues, including tyrosines 1173 and 1148 (Dc63), was employed [20]. Additional mutants were obtained by site-directed mutagenesis with substitution of tyrosine residues (Y1173, Y1148 and Y1068) with phenylalanine [21], generating single (F1173), double (F1173/1148) and triple (F1173/1148/1068) point-mutated receptors. Wild-type and mutant EGFRs were then transfected into NIH-3T3 cells by calcium phosphate precipitation. All cell clones were maintained at 37 °C in a humidified atmosphere of air/5 % CO_2 (19:1) in Dulbecco's modified Eagle's medium (DMEM) containing 10 % fetal calf serum (FCS), penicillin (100 units/ml) and streptomycin (100 µg/ml). Subculture (1:5 split ratio) was done when cells approached 95 % confluence.

Phosphorylation *in vivo* and immunoprecipitation of the EGFR

Cells (1×10^6) expressing either wild-type or mutant receptors were plated in 60 mm dishes and cultured in complete DMEM. After 8 h the cells were transferred to 2 ml of DMEM without phosphate containing 2 % FCS and 2 mCi of $[^{32}P]P_i$ (NEN), and incubated overnight. Duplicate dishes for each clone were then either stimulated with 100 nM-EGF for 10 min or immediately lysed. Each dish was lysed in 1 ml of EGFR lysis buffer (20 mM-Hepes, pH 7.5, 1 % Triton X-100, 10 % glycerol, 2 mM EDTA, 1 % aprotinin, 10 mM-sodium orthovanadate and 10 mM-sodium pyrophosphate) and clarified by centrifugation for 30 min at 10000 *g* and 4 °C. Equal amounts of lysates were incubated with either an anti-EGFR polyclonal antibody (Ab2913 [22]) or an anti-phosphotyrosine monoclonal antibody conjugated to Sepharose (Amersham [23]). Immunoprecipitation with the two antibodies was performed either as previously described [22] or according to the manufacturer's recommendation [23]. The pellets were finally resuspended in 50 µl of Laemmli buffer and analysed by SDS/PAGE (8.5 % gels) and autoradiography [24,25].

Tyrosine phosphorylation of PLC γ *in vivo*

Cells grown to subconfluence were incubated overnight in serum-free medium and then exposed to EGF (50 nM) for 10 min at 37 °C. Lysis was performed in a buffer containing 1 % Triton X-100, 50 mM-Hepes, pH 7.5, 150 mM-NaCl, 10 % glycerol, 1.5 mM- $MgCl_2$, 5 mM-EGTA, 2 mM-sodium orthovanadate, 2 mM-phenylmethanesulphonyl fluoride, 50 mg of aprotinin/ml and 10 mM-sodium pyrophosphate. Parallel samples of lysates were either immunoprecipitated with either anti-phosphotyrosine or anti-EGFR antibodies, or directly fractionated by SDS/PAGE and blotted on to nitrocellulose (see above) and revealed by ^{125}I -Protein A (Amersham) [26]. The antibodies used were: (1) a mixture of six monoclonal antibodies directed against PLC γ ([27]; kindly provided by Dr. S. G. Rhee); (2) a commercially available monoclonal anti-phosphotyrosine antibody (Upstate Biotechnology), and (3) a rabbit anti-peptide antibody against the EGFR C-terminus [26].

$[Ca^{2+}]_i$ measurements

Measurement of $[Ca^{2+}]_i$ was performed using the fluorescent dye fura-2 [28]. Suspensions of the various cell clones were obtained by a brief trypsin/EDTA treatment (0.005 % trypsin/0.9 mM-EDTA in phosphate-buffered saline) of monolayers from four 100 mm Petri dishes. The cells were washed once with culture medium and then resuspended (5×10^6 cells/ml) in the incubation buffer (Krebs-Ringer-Hepes, KRH) containing (mmol/l): NaCl, 125; KCl, 5; $MgSO_4$ and KH_2PO_4 , 1.2; $CaCl_2$, 2; glucose, 6; Hepes-NaOH, 25, pH 7.4. Loading with fura-2 was achieved by cell incubation at 37 °C for 30 min with 5 µM-fura-2/AM. The cell suspensions were then diluted to 10 ml with KRH and maintained at room temperature until processed. For fluorimetric measurements, 1 ml of the suspension was centrifuged at 10000 *g* for 3 s and the pellet was resuspended in 1.5 ml of warm KRH. The cells were then transferred to a cuvette in a thermostatically controlled holder (37 ± 2 °C), and the fluorescence signal was measured under continuous stirring. Calibration of the fluorescence signals in terms of $[Ca^{2+}]_i$ was done by using a K_d for the fura-2- Ca^{2+} interaction of 225 nM [28]. The results presented are representative of at least three separate experiments.

Membrane potential

Changes in membrane potential were studied using the slow-response fluorescent dye bis-oxonol, as previously reported [29,30]. Cell suspensions, prepared as described for $[Ca^{2+}]_i$ measurements, were washed once with KRH. Appropriate portions were then transferred to the fluorimeter cuvette and 100 nM-bis-oxonol was added from a 100-fold concentrated solution in dimethyl sulphoxide. The cells were allowed to equilibrate with the dye for approx. 10 min before stimulation with EGF. Downward or upward deflections of the fluorescence signal represent hyper- or de-polarizations respectively.

Inositol phosphate (InsP) generation

Cells were plated in 35 mm Petri dishes and grown in DMEM containing 10 % FCS until 70 % confluence. Labelling with *myo*- $[^3H]$ inositol was achieved by incubating cell monolayers with the radioisotope (5 µCi/ml) in an inositol-poor medium (final [inositol] = 10 µM) for 24–36 h. Incubation media were then removed and the monolayers were preincubated for 5 min in KRH, with 10 mM-LiCl replacing equimolar quantities of NaCl, after which EGF was added (10 nM). After 5 min, incubations were stopped by the addition of 2 ml of ice-cold 10 % trichloroacetic acid. In additional experiments, longer incubations (10 and 20 min) were used. Acid-treated dishes were placed on ice, and water-soluble radioactivity was extracted for 30 min. The whole supernatant was transferred to glass tubes and washed five times with 1 vol. of diethyl ether. Samples were brought to alkaline pH (~ 8.5) by the addition of a small (50 µl) volume of 3 M-Tris, then ion-exchange resin was added to each sample, which was then washed eight times with 60 mM-ammonium formate/5 mM-disodium tetraborate (glycero-phosphoinositol buffer). InsPs were eluted together by applying 3 ml of 1.7 M-ammonium formate, pH 3.7. A 2.5 ml portion of the supernatant was transferred to scintillation vials and counted for radioactivity after mixing with 16 ml of Packard Hionicfluor. For additional detail, see [29].

Materials

Serum and culture media were from Flow Laboratories; *myo*- $[^3H]$ inositol from Amersham International; EGF (receptor grade) and fibroblast growth factor (FGF) from Boehringer-Mannheim; fura-2 from Calbiochem; and bis-oxonol from

Molecular Probes. Other chemicals were reagent grade, supplied by the sources indicated previously [29,30].

RESULTS

Fig. 1 illustrates the various NIH-3T3 clones employed in this work. The deletion mutant Dc19 was engineered in order to remove the distal EGFR tyrosine autophosphorylation site located in the receptor tail, as well as the surrounding amino acids. In a few experiments another deletion mutant (Dc63, missing the two distal tyrosine residues; receptor density 1.3×10^5 /cell) was also employed [20]. Point mutants were obtained by substitution with phenylalanine residues of one (F1173), two (F1173/1148) or three (F1173/1148/1068) C-terminal tyrosines in the context of the full-length receptor [21]. Expression of mutated EGFRs in the cell clones that were isolated was found to be variable. This was expected to generate problems in the comparison of results obtained with different clones. In order to make comparisons possible, the mutated

clones that we have employed were chosen to have EGFR densities similar ($< 25\%$ difference) to those observed in the two wild-type EGFR clones, T17 and pC011 (see Fig. 1) that were employed as controls.

Receptor and PLC γ phosphorylation

The state of phosphorylation of the various EGFRs was investigated in [32 P]P $_i$ -labelled cells, stimulated or not with EGF for 10 min and then solubilized. Immunoprecipitations were then carried out using lysates containing dissimilar amounts of protein but the same numbers of EGFRs, as revealed by binding experiments. Two antibodies were employed, against either the receptor itself or phosphotyrosine, and the precipitates were separated by SDS/PAGE and revealed by autoradiography. The results obtained with the anti-EGFR antibody should provide data on the total phosphorylation of the receptor occurring after EGF treatment at both tyrosine and serine/threonine sites, whereas those obtained with the anti-phosphotyrosine antibody should reflect more specifically receptor autophosphorylation.

As shown in Fig. 2, no difference was observed between total phosphorylation (as revealed by the anti-EGFR immuno-

Fig. 1. Structure of the wild-type and mutated EGFRs

N and C mark N- and C-termini, and replacements of Y with F indicate the tyrosine → phenylalanine mutations at the indicated sites. The numbers to the right refer to the density (number/cell) of the EGFR expressed in the clones employed, revealed by Scatchard analysis of EGF binding experiments [21].

Fig. 2. EGF-induced phosphorylation and autophosphorylation of wild-type and mutated EGFRs *in vivo*

The various clones were labelled with [32 P]P $_i$ overnight and were then incubated without or with 100 nM-EGF for 10 min before lysis, immunoprecipitation and SDS/PAGE of the precipitates. The gels to the left are of anti-EGFR immunoprecipitates, and those to the right are of anti-phosphotyrosine immunoprecipitates. Deletion mutants Dc19 (lanes 3 and 4) and Dc63 (lanes 5 and 6) are illustrated in (a), and point mutants F1173 (lanes 3 and 4), F1173/1148 (lanes 4 and 6) and F1173/1148/1068 (lanes 7 and 8) in (b). Wild-type EGFR is shown in lanes 1 and 2. Odd- and even-numbered lanes were without and with EGF stimulation respectively.

Fig. 3. Expression and tyrosine phosphorylation *in vivo* of PLC γ

The two cell clones used, wild-type (lanes 1 and 2) and mutated (lanes 3 and 4) were pC011 and F1068/1148/1173 respectively. Cells were treated without or with EGF (50 nM) at 37 °C for 10 min, and lysates were prepared. (a) Tyrosine phosphorylation of PLC γ by wild-type or mutated receptors. Total cell lysates (4 mg) were immunoprecipitated with an excess of monoclonal anti-phosphotyrosine antibodies. Immunoprecipitates were run on SDS/8.5% polyacrylamide gels, blotted on nitrocellulose and treated with monoclonal antibodies against PLC γ . The autoradiogram was exposed for 1 h. (b) Total cell lysates (100 μ g) treated by immunoblot with the anti-PLC γ antibodies; exposure time 2 h. (c) Total cell lysates (100 μ g) treated by immunoblot with an anti-EGF receptor antibody; exposure time 4 h. (d) Densitometric scans of the autoradiograms in (a), (b) and (c) were performed on exposures taken in the linear range of signal intensity. Results shown are relative phosphotyrosine values in PLC γ normalized on the basis of EGFR levels in the two clones. ■, Wild-type; ▨, mutant. Results consistent with those illustrated here were obtained in three independent experiments.

Fig. 4. Inositol phosphate generation in cell clones expressing wild-type and mutated EGFRs, exposed to EGF or FCS

Each bar group refers to a single clone and illustrates total *InsP* radioactivity recovered in cell monolayers that were untreated (■) or exposed for 5 min to either EGF (10 nM, □) or FCS (10%, ▨). In order to facilitate the comparison, the clones are arranged in two groups characterized by similar EGFR expression. From left to right: the wild-type control T17 followed by the double and triple mutated EGFR clones; then the wild-type control pC011 followed by the Dc19 deletion and the single point mutant. Note that the responses to FCS are similar in all clones, whereas those to EGF are higher in controls compared with the mutated clones with similar EGFR expression, and that the decrease is greater in the double and especially the triple-mutated clones with respect to the clones lacking a single tyrosine residue. Results shown are averages of three independent experiments.

precipitates) of the wild-type, the Dc19 and Dc63 deletion mutants and all of the point-mutated receptors. In the phosphotyrosine immunoprecipitates, no appreciable decreases in phosphorylation were observed in the single point mutant (F1173) or in the equivalent deletion mutant (Dc19). In the other mutants, involving removal of two or all three autophosphorylation sites, tyrosine phosphorylation was decreased, but still appreciable. It seems reasonable to assume that, especially in the triple point mutant, tyrosine phosphorylation occurred at additional sites such as tyrosine-1086 [31].

In order to investigate tyrosine phosphorylation of *PLCγ*, lysates obtained from clones, one (pC011) expressing the wild-type EGFR and the other (F1173/1148/1068) expressing the triple-point-mutated EGFR, were employed. Figs. 3(b) and 3(c) show that the concentration of *PLCγ* (revealed by SDS/PAGE immunoblotting with specific antibodies [25]) was very similar in the two clones, whereas the concentration of EGFR (measured by the same technique) was distinctly greater in the mutant, consistent with the data of Fig. 1. In contrast, the EGF-induced tyrosine phosphorylation of the lipolytic enzyme was markedly higher in the wild-type pC011 clone (Fig. 3a). Accurate densitometry of these data revealed the phosphotyrosine-positive fraction of *PLCγ*, normalized on the basis of EGFR expression, to be decreased in the mutant-EGFR-bearing clone to ~25% of that in the wild-type clone (Fig. 3d).

InsP generation, $[Ca^{2+}]_i$ and membrane potential

Compared with autophosphorylation, the decrease in *InsP* generation observed in the clones expressing the various mutated

Fig. 5. Increase in $[Ca^{2+}]_i$ induced by EGF and FGF in cell clones expressing either wild-type or mutated EGFR receptors

Cell suspensions loaded with the fluorescent Ca^{2+} indicator fura-2 were continuously monitored. EGF (10 nM) and FGF (5 nM) additions are marked by arrows. Note that in both panels [a showing clones with $(1.2\text{--}1.5) \times 10^5$ EGFR, b showing clones with $(3.5\text{--}4) \times 10^5$ EGFR], the responses to FGF were similar, whereas those to EGF were decreased in the mutants, particularly in those with double and triple tyrosine → phenylalanine mutations.

receptors were even more pronounced (Fig. 4). With the mutants lacking the distal tyrosine (Dc19 and Dc63 deletion mutants), the *InsPs* released during a 5 min incubation with optimal EGF concentrations (10 nM) represented only a fraction (65–70%) of those released by the wild-type clone expressing a similar number of EGFRs (pC011), whereas with the F1173/1148 mutant the response was even lower and with the triple mutant it was barely appreciable (~40% and 12% respectively of the control with similar levels of EGFR expression, in this case the T17 clone). Similar results were obtained with longer EGF treatments (15 and 30 min; results not shown). In order to make sure that the differences described above really were due to the different EGFRs expressed and not dependent on vastly different *InsP* generation potentials of the various cell clones, control experiments were carried out using 10% FCS instead of EGF. As shown in Fig. 4, some variability was also observed with FCS; however, this variability (max. = 15%) was much smaller than that observed with the growth factor.

The effects of the various EGFR mutations on *InsP* generation were further investigated indirectly by assaying the initial changes in $[Ca^{2+}]_i$ (first few minutes) induced by EGF. These changes are in fact known to be largely sustained by Ca^{2+} release from intracellular stores triggered by *InsP*₃, the second messenger generated via the hydrolysis of phosphatidylinositol 4,5-bisphosphate [14,30,32–35]. As shown in Figs. 5(a) and 6, the

Fig. 6. Concentration-dependence of the increases in $[Ca^{2+}]_i$ triggered by EGF (●) and FGF (○) in cell clones expressing either the wild-type or a mutated EGFR

Each panel shows to a single clone, as indicated. Values are initial peak increases, expressed as percentages of the maxima observed after FGF addition. In absolute terms, the latter values varied from 360 to 440 nM- $[Ca^{2+}]_i$ (increase above resting level) in the F1173 and pC011 clones respectively.

Fig. 7. Effect of EGF on membrane potential in cell clones expressing wild-type or mutated EGFR

Cells suspensions loaded with the fluorescent voltage indicator bis-oxonol were continuously monitored. Results are expressed in mV. Downward deflections represent hyperpolarizations. Additions of EGF (10 nM) are marked by arrows. Traces in (a)–(c) and those in (d)–(f) refer to clones expressing similar densities of EGFR.

responses were decreased, although moderately (20–36%), in both the deletion and point mutants lacking the distal tyrosine residue. With the double mutation the decrease was more marked (50–70%), and with the additional replacement of the proximal tyrosine-1068 residue the response was almost completely abolished (Figs. 5b and 6). As in the phosphoinositide hydrolysis experiments, in order to check for general $[Ca^{2+}]_i$ responsiveness, the various clones expressing either the wild-type or the mutant EGFR were expressed to a different stimulus, in this case another growth factor, FGF, administered at concentrations from 10^{-10} to 10^{-7} M [36]. As shown in Figs. 5 and 6, in all the clones

investigated the $[Ca^{2+}]_i$ responses to FGF were markedly similar to each other in size, time course and concentration-dependence, indicating that the decreases observed with EGF were not due to general hyporesponsiveness of the mutant EGFR-bearing cells. Moreover, comparison of the EGF and FGF concentration-response curves of Fig. 5 demonstrated that these decreases were not so much due to a loss of EGF potency, but rather to a marked decrease in EGF efficacy.

Results similar to those of $[Ca^{2+}]_i$ were obtained also by investigating the effect of EGF on membrane potential. The large hyperpolarization induced in the clones expressing the wild-type EGFR (due to the activation of Ca^{2+} -dependent K^+ channels [29]) was in fact decreased in those cells expressing the mutant receptors (Fig. 7), in parallel with the decreased $[Ca^{2+}]_i$ responses. Again, no substantial difference between the Dc19 deletion and the corresponding F1173 point mutant was observed.

DISCUSSION

The present work was carried out to investigate whether, and to what extent, the C-terminal autophosphorylation sites of the EGFR control the process of activation of PLC γ , a substrate of the EGFR tyrosine kinase. Recently, two reports have appeared implicating the autophosphorylation process as a positive regulator of the physical association between the EGFR and the lipolytic enzyme [16,17], but the precise role of the various tyrosine residues involved (at positions 1173, 1168 and 1048) and of the adjacent amino acid sequences was not established. Moreover, the only activity data available (concerning, however, not directly the enzyme-catalysed reaction, but one of its consequences, the increase in $[Ca^{2+}]_i$ in EGF-treated cells) were in apparent contrast with those on the EGFR–PLC γ association. In fact, in cells expressing EGFRs with the C-terminal tail deleted up to residue 991, the EGF-induced response was found to be increased, and not decreased, relative to the controls [19]. Taken together with the previous data on the EGFR–PLC γ

association, these results could have been interpreted as a suggestion that the two processes (association with and phosphorylation of PLC γ by EGFR) are separate and independent. The results that we have now obtained demonstrate that this is probably not the case.

The reported disagreement between the association and activity data discussed above [16,17,19] may have been only apparent, for two possible reasons. Large deletions of the C-terminal tail, such as those investigated by Chen *et al.* [19], could have modified unspecifically the process of enzyme-substrate interaction, in particular by eliminating the link between association and phosphorylation which may exist between the intact EGFR and the enzyme. Moreover, the activity studies of Chen *et al.* [19] were carried out with a mutated cell clone expressing almost twice as many EGFRs as the control, wild-type clone. We and others [34,37] had already reported that the size of the response initiated by PLC γ activation (i.e. phosphoinositide hydrolysis as well as increases in $[Ca^{2+}]_i$ and V_m) depends profoundly on the number of EGFRs expressed by the cells. This is confirmed also in the present work, where the three types of responses triggered by EGF in the two wild-type clones used as controls, T17 and pC011 (expressing 4×10^5 and 1.2×10^5 EGFR/cell respectively), differed from each other by 3–4-fold.

In order to cope with these problems, on the one hand we have employed NIH-3T3 fibroblasts expressing mutated EGFRs only slightly different from the wild-type receptor (substitution of one, two or three tyrosine with phenylalanine residues or small deletions); on the other hand, the mutated clones were chosen based on their EGFR density, which was close ($< 25\%$ difference) to that of a wild-type clone (see Fig. 1). Experimental-to-control comparisons could thus not be affected by major differences in receptor expression. However, receptor expression is not the only property of the clones that can, at least in principle, become limiting in the generation of physiological responses. Among other at least potentially important properties we can list the PLC γ and phosphatidylinositol 4,5-bisphosphate content of the cells, the density of $InsP_3$ receptors and plasma membrane channels (both cation- and K^+ / Ca^{2+} -dependent channels) activated on EGF treatment, and the size of the rapidly exchanging intracellular Ca^{2+} pool(s). The PLC γ content was investigated directly by Western blotting in the wild-type and in the triple-mutated clones, and was found to be very similar in both. This is in contrast with EGF-induced tyrosine phosphorylation, which was drastically decreased in the mutant. With the other clones no direct evidence is available, and the same is true for the other properties listed above. It should be mentioned, however, that all the clones investigated responded in a similar fashion to the controls when treated with other agents (i.e. FCS and FGF) which are known to activate the investigated responses by the same general mechanisms as does EGF, and which were employed as controls for phosphoinositide hydrolysis and $[Ca^{2+}]_i$. We conclude that the general responsiveness of the various clones was similar to the control, and that the differences observed with EGF were due, at least primarily, to the mutations in the EGFR C-terminal tail.

The results that we have obtained demonstrate clearly a positive regulatory role of EGFR C-terminal tyrosine residues in PLC γ activation. In all our mutants the responses dependent on the enzyme were in fact decreased: to a moderate extent when only the distal tyrosine was missing, whether by point mutation or deletion, markedly (50–70%) in the double mutant and very severely (80–90%) in the triple mutant. The activity data for the triple mutant agree well with those on PLC γ tyrosine phosphorylation, which revealed an approx. 75% decrease. This result concurs with recent conclusions that phosphorylation of the lipolytic enzyme is directly responsible for its activation

[38–40]. Compared with the EGF-triggered activities investigated, autophosphorylation of EGFR was decreased to a smaller extent. This result is not surprising, in view of the recently identified additional tyrosine residues that can be phosphorylated and can therefore account for the residual phosphorylation observed in the triple point mutant [18,31].

With regard to the mechanism by which mutations affect EGFR functioning, previous studies with our various mutants had revealed no changes in their affinity for EGF [20,21]. This result is in agreement with the present observation that the apparent potency of the growth factor was essentially unchanged, while its efficacy was decreased. Moreover, the stepwise increase in inhibition observed with our single, double and triple mutants suggests that the various tyrosine residues play similar roles, i.e. they are not important as the members of specific amino acid sequences, but, after phosphorylation, they confer to the C-terminal tail strong strategically located negative charges that might be needed to open the access of PLC γ to a specific binding site. The latter would be responsible for the association of PLC γ with the EGFR, envisaged as a preliminary step in the process of phosphorylation (see also [16,17]). This conclusion appears to be consistent with recently reported results on a phosphorylated EGFR C-terminal peptide which was found to compete with the entire receptor for PLC γ binding [17]. The sequence in the EGFR tail that functions as the binding site for PLC γ has not yet been identified. Our data with the deletion mutant seem to exclude the distal 19 amino acids, and preliminary results with a mutant lacking the 63 distal amino acids seem to exclude the additional sequence as well. A possible candidate is the EGFR (973–991)-peptide reported by Chen *et al.* [19] to be needed for eliciting responses in $[Ca^{2+}]_i$ in cells expressing largely deleted EGFR. This possibility, however, remains to be investigated.

PLC γ is not the only EGFR substrate known to associate with the receptor to form a complex. This has also been reported for the GTPase-activating protein of the *ras* proto-oncogene (GAP) and for a few other proteins, all endowed with similar sequences (denominated SH2 and SH3) which are thought to participate in the complex formation [1,2,7,8,17]. Thus it seems likely that the positive role of autophosphorylation that we have described here exists not only for the lipolytic enzyme but for other EGFR substrates as well. Moreover, a similar situation could exist at other growth factor receptors which are also endowed with both intrinsic tyrosine kinase activity and autophosphorylated tyrosine residues. Because of this possible wide-ranging application, the so far largely unexplored role of the EGF receptor C-terminal tail in cell physiology deserves further investigation.

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REFERENCES

1. Carpenter, G. & Cohen, S. (1990) *J. Biol. Chem.* **265**, 7709–7712
2. Ullrich, A. & Schlessinger, J. (1990) *Cell* **61**, 203–211
3. Pandiella, A., Beguinot, L., Vicentini, L. M. & Meldolesi, J. (1989) *Trends Pharmacol. Sci.* **10**, 411–414
4. Heldin, C. H. & Westermark, B. (1990) *Cell. Regul.* **1**, 555–566
5. Sawyer, S. T. & Cohen, S. (1985) *J. Biol. Chem.* **260**, 8233–8236

6. Rossomando, A. J., Payne, D. M., Weber, M. J. & Sturgill, T. W. (1989) *Proc. Natl. Acad. Sci. U.S.A.* **86**, 6940–6943
7. Molloy, C. J., Bottaro, D. P., Fleming, T. P., Marshall, M. S., Gibbs, J. B. & Aaronson, S. A. (1989) *Nature (London)* **342**, 711–714
8. Ellis, C., Moran, M., McCormick, F. & Pawson, T. (1990) *Nature (London)* **343**, 377–380
9. Wahl, M. I., Daniel, T. U. & Carpenter, G. (1988) *Science* **241**, 968–970
10. Wahl, M. I., Nishibe, S., Suh, P. G., Rhee, S. G. & Carpenter, G. (1989) *Proc. Natl. Acad. Sci. U.S.A.* **86**, 1568–1572
11. Nishibe, S., Wahl, M. I., Rhee, S. G. & Carpenter, G. J. (1989) *J. Biol. Chem.* **264**, 10335–10338
12. Margolis, B., Rhee, S. G., Feloel, S., Mervic, M., Lyall, R., Levitzky, A., Ullrich, A., Zilberstein, A. & Schlessinger, J. (1989) *Cell* **57**, 1101–1107
13. Meisenholder, S., Suh, P. G., Rhee, S. G. & Hunter, T. (1989) *Cell* **57**, 1109–1122
14. Berridge, M. J. & Irvine, R. F. (1989) *Nature (London)* **312**, 315–321
15. Tsai, M. H., Yu, C. L. & Stacey, D. W. (1990) *Science* **250**, 82–86
16. Margolis, B., Bellot, A. M., Honeger, A. M., Ullrich, A., Schlessinger, J. & Zilberstein, A. (1990) *Mol. Cell. Biol.* **10**, 435–441
17. Margolis, B., Li, N., Koch, A., Mohammadi, M., Hurwitz, D. R., Zilberstein, A., Ullrich, A., Pawson, T. & Schlessinger, J. (1990) *EMBO J.* **9**, 4375–4380
18. Walton, G. M., Chen, W. S., Rosenfeld, M. G. & Gill, G. N. J. (1990) *J. Biol. Chem.* **265**, 1750–1754
19. Chen, W. S., Lazar, C. S., Lund, K. A., Welsh, J. B., Chang, C. P., Walton, G. M., Der, C. J., Wiley, H. S., Gill, G. N. & Rosenfeld, M. G. (1989) *Cell* **59**, 33–43
20. Velu, T. J., Vass, W. C., Lowy, D. R. & Beguinot, L. (1989) *Mol. Cell. Biol.* **9**, 1772–1778
21. Helin, K., Velu, T., Martin, P., Vass, B., Allevato, G., Lowy, D. R. & Beguinot, L. (1991) *Oncogene*, in the press
22. Beguinot, L., Hanover, J. A., Ito, S., Richert, N. D., Willingham, M. C. & Pastan, I. (1985) *Proc. Natl. Acad. Sci. U.S.A.* **82**, 2774–2778
23. Frackelton, A. R., Jr., Tremble, P. M. & Williams, L. T. (1984) *J. Biol. Chem.* **259**, 7909–7915
24. Laemmli, U. K. (1970) *Nature (London)* **227**, 680–685
25. Towbin, H., Staehelin, T. & Gordon, J. (1979) *Proc. Natl. Acad. Sci. U.S.A.* **76**, 4350–4354
26. Di Fiore, P. P., Segatto, O., Lonardo, F., Fazioli, F., Pierce, J. H. & Aaronson, S. A. (1990) *Mol. Cell. Biol.* **10**, 2749–2756
27. Suh, P.-G., Ryu, S. H., Cho, K. S., Lee, K. Y. & Rhee, S. G. (1988) *J. Biol. Chem.* **263**, 14497–14504
28. Grynkiewicz, G., Poenie, M. & Tsien, R. Y. (1985) *J. Biol. Chem.* **260**, 3440–3450
29. Pandiella, A., Magni, M., Lovisolo, D. & Meldolesi, J. (1989) *J. Biol. Chem.* **263**, 12914–12921
30. Pandiella, A., Beguinot, L., Velu, T. J. & Meldolesi, J. (1988) *Biochem. J.* **254**, 223–228
31. Margolis, B. L., Lax, I., Kris, R., Dombalagian, M., Honegger, A. M., Howk, R., Gival, D., Ullrich, A. & Schlessinger, J. (1989) *J. Biol. Chem.* **264**, 10667–10671
32. Pandiella, A., Malgaroli, A., Meldolesi, J. & Vicentini, L. (1987) *Exp. Cell Res.* **170**, 175–185
33. Hepler, S. R., Nahakata, N., Lovenberg, T. S., Di Giuseppe, J., Herman, B., Earp, H. S. & Harden, T. (1987) *J. Biol. Chem.* **262**, 2959–2966
34. Wahl, M. I., Sweatt, J. D. & Carpenter, G. (1987) *Biochem. Biophys. Res. Commun.* **142**, 688–695
35. Pike, L. J. & Eakes, A. T. (1987) *J. Biol. Chem.* **262**, 1644–1651
36. Pandiella, A., Magni, M. & Meldolesi, J. (1989) *Biochem. Biophys. Res. Commun.* **163**, 1325–1331
37. Pandiella, A., Lehtvaslaiho, H., Magni, M., Alitalo, K. & Meldolesi, J. (1989) *Oncogene* **4**, 1299–1305
38. Margolis, B., Zilberstein, A., Franks, C., Felder, S., Kremer, S., Ullrich, A., Rhee, S. G., Skorecki, K. & Schlessinger, J. (1990) *Science* **248**, 607–610
39. Nishibe, S., Wahl, M. I., Hernandez-Sotomayor, S. M. T., Tonks, N. K., Rhee, S. G. & Carpenter, G. (1990) *Science* **248**, 1253–1256
40. Goldschmidt-Clarmont, P. J., Kim, J. W., Machesky, L. M., Rhee, S. G. & Pollard, T. D. (1991) *Science* **251**, 1231–1233

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