The EMBO Journal vol.7 no.10 pp.3045–3052, 1988

Biological activities of EGF-receptor mutants with individually altered autophosphorylation sites

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Communicated by J.Schlessinger

***In vitro* site-directed mutagenesis was used to replace individually the three known autophosphorylation sites of the epidermal growth factor (EGF)-receptor (i.e. Tyr1173, Tyr1148 and Tyr1068) by phenylalanine, a residue which cannot serve as a phosphate acceptor site. In another mutant, Tyr1173 was substituted by a serine residue. The cDNA constructs encoding either mutant or wild-type EGF-receptors were transfected into NIH-3T3 cells devoid of endogenous EGF-receptors. The mutant receptors were expressed on the cell surface and displayed typical high- and low-affinity binding sites for [^{125}I]EGF. Phorbol ester (PMA) modulated the binding affinity of wild-type and mutant receptors in a similar manner. Mutant EGF-receptors exhibited EGF-dependent tyrosine kinase activity leading to self-phosphorylation and phosphorylation of exogenous substrates both *in vitro* and in living cells. The internalization and degradation of EGF-receptors were not affected by the mutations. Cells expressing mutant EGF-receptors became mitogenically responsive to EGF, indicating that none of the vital functions of the EGF-receptor were critically impaired by the loss of individual autophosphorylation sites. Maximal mitogenic stimulation correlated with the number of wild-type or mutant receptors per cell, highly expressing cells showing higher maximal stimulation. However, the dose–response curves of cells expressing mutant receptors were slightly shifted to lower concentrations of EGF, rendering the cells mitogenically responsive to lower doses of EGF than cells expressing normal EGF-receptor at similar expression levels. Basal [^{3}H]-thymidine incorporation in the presence of 0.5% calf serum was consistently higher for cells expressing mutant receptors, while the response to stimulation with 10% calf serum was not affected.**

Key words: EGF-receptor/phosphorylation/receptor mutants

Introduction

The mitogenic response to epidermal growth factor (EGF) is mediated by a cell surface receptor called the EGF-receptor (reviewed in Carpenter and Cohen, 1979; Schlessinger, 1986). The EGF-receptor (EGF-R) is a 170 000 dalton glycoprotein whose intrinsic protein tyrosine kinase activity is regulated by EGF. Tyrosine phosphorylation of cellular substrates is essential for signal transduction, as various mutant EGF-Rs devoid of kinase activity are unable to elicit mitogenesis and to stimulate various effects of EGF (Livneh *et al.*, 1986, 1987; Chen *et al.*, 1987; Honegger *et al.*, 1987a,b; Moolenaar *et al.*, 1988). In addition to the phosphorylation of various cellular substrates, the EGF-R undergoes rapid autophosphorylation, and three autophosphorylation sites have been identified in the C-terminal end of the intracellular domain of the EGF-R, i.e. Tyr1173, Tyr1148 and Tyr1068 (Downward *et al.*, 1985).

Autophosphorylation has been found to increase the kinase activity of protein tyrosine kinases such as the kinase of the insulin receptor (Rosen *et al.*, 1983), pp$^{60c\text{-}src}$ (Piwnica-Worms, 1987) and of the pp$^{120gag\text{-}fps}$ oncogene (Hansen-Meckling, 1987). Reports on the effect of autophosphorylation on the kinase activity of EGF-R are conflicting. Bertics and Gill (1985a,b) reported that EGF-R autophosphorylation increased the activity of receptor kinase, while Downward *et al.* (1985) could not find a change in the activity of the EGF-R kinase upon autophosphorylation. Deletions in the C-terminal region leading to the loss of one or more autophosphorylation sites were found in the v-*erbB* oncogene product, which is a truncated avian EGF-R (Downward *et al.*, 1984) and also in other transforming oncogenes with tyrosine kinase activity (reviewed in Yarden and Ullrich, 1988). To explore the role of receptor autophosphorylation in regulating the activity of the EGF-R, we have generated different EGF-R mutants in which each of the individual autophosphorylation tyrosine sites has been replaced by a phenylalanine residue. These mutants were tested for their ability to mediate the various responses to EGF. We found that the mutant receptors are as active as wild-type receptor in mediating various EGF responses. Moreover, cells expressing the mutant receptors appeared to be slightly more sensitive to EGF-induced mitogenesis than cells expressing wild-type receptors. In the accompanying paper we analyzed in detail and compared the kinetic properties of the protein tyrosine kinase of the various mutant receptors (Honegger *et al.*, 1988).

Results

To test the role of autophosphorylation in the regulation of the biological activity of the EGF-R, several EGF-R mutants were constructed in which individual autophosphorylation sites (Tyr1173, Tyr1148 and Tyr1068) were substituted by phenylalanine, a residue which cannot act as phosphate acceptor site. In another mutant, Tyr1173 was replaced by a serine residue which could serve as a phosphate acceptor site for serine- or threonine-specific protein kinases, but not for protein tyrosine kinases. We have also used a previously

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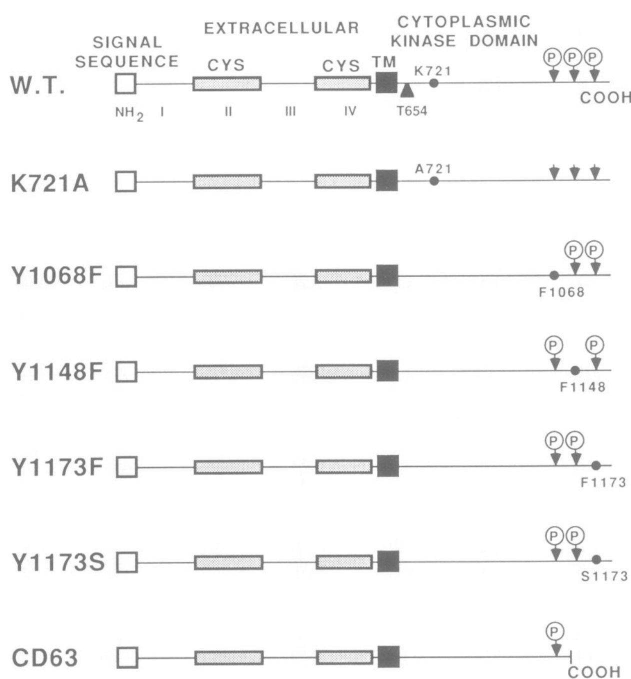

Fig. 1. Schematic representation of the EGF-R mutants. **(WT)** Wild-type human EGF-receptors; (HER); Cys denotes the two cysteine-rich domains; TM the transmembrane sequence; P the identified autophosphorylation sites Y1068, Y1148 and Y1173. **(K721A)** Point mutant of the EGF-R in which lysine 721 was replaced by an alanine residue resulting in the loss of enzymatic activity of the kinase. **(Y1068F, Y1148F, Y1173F)** Point mutants of individual autophosphorylation site tyrosines to phenylalanine residues. **(Y1173S)** Point mutant of tyrosine 1173 to a serine residue. **(CD63)** C-terminal truncation of 63 amino acids, deleting autophosphorylation sites Y1148 and Y1173.

described deletion mutant devoid of two autophosphorylation sites (Livneh *et al.*, 1986) and a kinase-negative receptor mutant in which Lys721 was replaced by an alanine residue (Honegger *et al.*, 1987a,b). The EGF-R cDNA constructs were cloned into a mammalian expression vector containing the SV40 early promoter to drive transcription of the construct and dihydrofolate reductase and neomycin resistance genes as selectable markers. Figure 1 shows a schematic summary of the receptor constructs described in this study. The wild-type and mutant EGF-R constructs were transfected into an NIH-3T3 cell line devoid of endogenous EGF-Rs. Geneticin (G418) was used to select resistant clones containing the transfected plasmids. The selected clones were screened for the expression of EGF-R by binding experiments with ^{125}I-labeled EGF and by immunoprecipitation with various human-specific anti-EGF-R antibodies.

For each construct several clones showing expression levels between 30 000 and 500 000 receptors/cell were analyzed. Anti-peptide antibodies against the N-terminal and the C-terminal ends of the human EGF-R were used to confirm the presence of the complete human EGF-R sequence (data not shown). Comparison of phosphopeptide maps of EGF-R autophosphorylated *in vitro* confirmed the loss of one individual autophosphorylation site in each of the mutant receptors (Honegger *et al.*, 1988). For the Y1148F mutant only low-expressing clones were obtained, and therefore some of the experiments done with cells expressing this mutant receptor could not be interpreted.

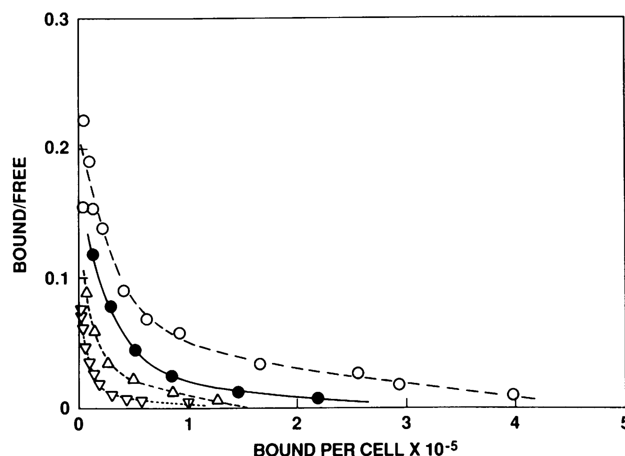

Fig. 2. Scatchard analysis of [^{125}I]EGF binding to intact cells expressing either wild-type or mutant EGF-Rs. Confluent cells in 24-well Costar dishes were incubated for 1 h at room temperature with different concentrations of [^{125}I]EGF. Binding results were plotted as Scatchard plots and fitted by a two-site model to obtain receptor numbers and ligand dissociation constants (Table I). Nonspecific binding was determined by parallel binding experiments to either parental cells which are devoid of EGF-Rs or by binding experiments in the presence of excess cold ligand. Cell lines expressing various amounts of EGF-R on their cell surface are shown in order to emphasize that the nonlinearity of the Scatchard plots is independent of receptor number. HER wild-type receptor (●); mutant receptor Y1068F (○); mutant receptor Y1148F (▽); and mutant receptor Y1173F (△).

Table I. Effect of PMA on [^{125}I]EGF binding to cells expressing EGF-R mutants

	Control				+ PMA	
	$K1$ (nM)	n 1	$K2$ (nM)	$n2$	K (nM)	n
HER	0.6	46 600	40	298 000	38	320 000
Y1068F	0.4	35 000	20	460 000	47	520 000
Y1148F	0.4	13 000	41	82 000	22	61 000
Y1173F	0.5	17 000	18	243 000	20	200 000
Y1173S	0.7	11 000	30	94 000	24	69 000

Binding parameters are determined from Scatchard plots which were fitted either by one-site or two-site models to obtain receptor number (n) and ligand dissociation constants (K). [^{125}I]EGF binding was determined at room temperature after preincubation of the cells with 100 nM PMA for 30 min at 37°C as described in Materials and methods. In the presence of 100 nM PMA, the amount of high affinity receptors was $<0.5\%$ while in the absence of PMA the amount of high affinity receptor is 5–15% of total receptor numbers.

Mutant receptors have normal binding properties

The binding affinity of [^{125}I]EGF to the EGF-R was not affected by the mutations which abolish individual autophosphorylation sites. Analyses of Scatchard plots of [^{125}I]EGF binding to cells expressing either wild-type or mutant EGF-R showed typical nonlinear plots for all cell lines (Figure 2). These results were interpreted as an indication for the existence of two classes of binding sites. Phorbol ester (PMA) selectively suppressed the high affinity component. Table I summarizes receptor numbers and binding constants for cells expressing either wild-type or mutant receptors in the absence or presence of PMA.

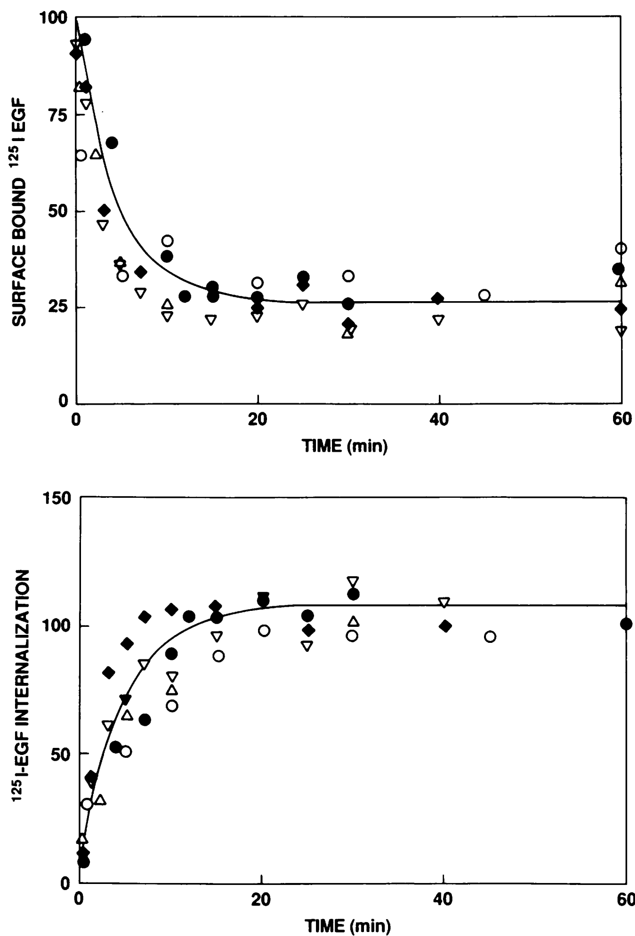

Fig. 3. Internalization of [^{125}I]EGF and EGF-R down-regulation. Confluent cells in 24-well Costar dishes were incubated with 100 ng/ml [^{125}I]EGF for 90 min at 4°C, then warmed up to 37°C. After different incubation times at 37°C, the cells were washed and surface associated ligand separated from internalized ligand by an acid-wash procedure and counted separately in a counter. (**A**) [^{125}I]EGF bound to the cell surface (normalized acid-dissociable radioactivity) as a function of incubation time at 37°C. (**B**) [^{125}I]EGF internalized (normalized acid-resistant radioactivity) as a function of incubation time at 37°C. Maximal internalization at 60 min corresponded to 100 ± 15% of the total initial binding for all cell lines. HER (●); Y1068F (○); Y1148F (▽); Y1173F (△); Y1173S (▼); CD63 (◆).

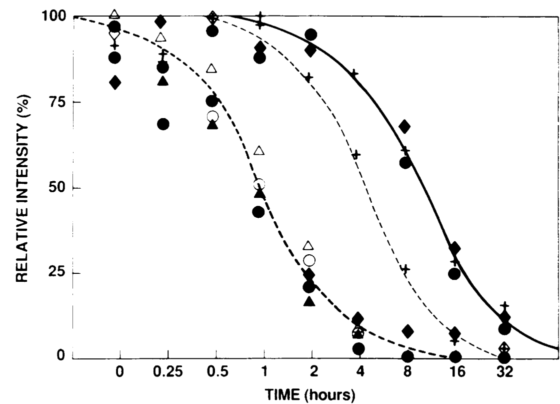

Fig. 4. Biosynthetic half-life of wild-type or mutant EGF-Rs in the presence or absence of EGF. Cells were labeled overnight with [^{35}S]methionine, then grown in normal medium containing 10% calf serum with or without addition of 200 ng/ml EGF. After different chase times up to 32 h, the cells were lysed, the EGF-R immunoprecipitated with monoclonal antibodies (mAb 108.1) and analyzed by SDS−PAGE. Autoradiographs of the gels were densitometrically scanned and the relative band intensities were plotted against the chase time. In the absence of EGF the half-life of the receptor was 11−13 h, while in the presence of EGF the half-life of wild-type receptors, the autophosphorylation site mutants and the C-terminal deleted mutant CD63 were between 30 and 60 min. For comparison, the half-life of the kinase negative mutant K721A is 6−7 h in the presence of EGF. Half-life of receptors in the absence of EGF (—); HER (●); K721A (+) and CD63 (◆). Half-life of receptors in the presence of EGF (---); HER (●); Y1068F (○); Y1148F (△); Y1173F (◇); CD63 (◆); and K721A (---),(+).

Mutant receptors undergo normal internalization and degradation

Occupied wild-type EGF-R is rapidly internalized and degraded, leading to the depletion of surface binding sites resulting in receptor 'down-regulation'. Eventually most of the internalized receptor molecules enter a degradative pathway, leading to a marked decrease of the receptor half-life in the presence of EGF. An EGF-R mutant lacking intrinsic kinase activity due to a point mutation of lysine 721 to an alanine residue does not show receptor down-regulation despite efficient internalization. It recycles to the cell surface rather than entering the degradative pathway after internalization (Honegger *et al.*, 1987b). In contrast, all mutant receptors tested, including the C-terminal deletion mutants, internalized [^{125}I]EGF with essentially the same kinetics as cells expressing wild-type EGF-Rs (Figure 3A). When cells were preincubated with ligand at 4°C and then warmed to 37°C, ~50% of the ligand bound to the cell surface entered the cells within the first 4−6 min. Loss of surface binding paralleled internalization, so that 50% of the surface binding site disappeared from the cell surface within the same time interval (Figure 3B).

When cells were labeled with [^{35}S]methionine, then chased in medium containing 10% serum and unlabeled methionine, wild-type receptor showed a half-life of 11−12 h. EGF, 200 ng/ml, added to the medium reduced the half-life of the EGF-R to ~1 h. Mutant receptors with altered autophosphorylation sites as well as the C-terminal deletion mutant had a half-life similar to the half-life of wild-type receptor. Moreover, in the presence of EGF the mutant receptors were rapidly degraded with kinetics similar to kinetics of degradation of wild-type receptors (half-life ~1 h) (Figure 4). This is in contrast to the kinase-negative mutant K721A, which has a long half-life even in the presence of EGF (Honegger *et al.*, 1987b and Figure 4). Mutant receptors Y1068F, Y1148F, Y1173F and Y1173S as well as the deletion mutant CD63 all behaved like wild-type receptor with respect to EGF-induced receptor down-regulation, indicating that lack of autophosphorylation of individual sites cannot account for the altered cellular routing of K721A.

Phosphorylation of mutant receptors in living cells

The transfected cells were starved, incubated with [^{32}P]orthophosphate and then exposed to either EGF or to PMA. In quiescent cells, EGF-R shows basal phosphorylation on serine and threonine residues, but no tyrosine phosphorylation was detected (Honegger *et al.*, 1987b). PMA stimulation leads to increased phosphorylation of the EGF-R on serine and threonine residues, while EGF causes stimulation of EGF-R phosphorylation on serine, threonine

and tyrosine residues. Figure 5 shows the effect of EGF and PMA on the phosphorylation of wild-type and mutant EGF-Rs in living cells. The mutant receptors clearly showed increased phosphorylation in response to stimulation by EGF or by PMA.

Tyrosine phosphorylation of EGF-R in living cells was studied by using anti-phosphotyrosine antibodies as a specific probe (Figure 6). Cells were stimulated with EGF or PMA, lysed in the presence of phosphatase inhibitors, and after immunoprecipitation with antibodies against human EGF-R analyzed by SDS−PAGE. After transfer to nitrocellulose, the samples were immunoblotted either with antibodies against EGF-R (as a measure of total receptor concentration) or with antibodies against phosphotyrosine (as a measure of phosphotyrosine content of EGF-R). In unstimulated cells, in cells treated with PMA and in EGF-stimulated cells expressing the kinase-negative mutant (K721A), tyrosine phosphorylation of the EGF-R was below the detection limit. All autophosphorylation site mutants showed enhanced tyrosine phosphorylation in response to EGF. The shift in the migration of the EGF-R band seen in the immunoblots using anti-EGF-R antibodies (Hunter and Cooper, 1981) indicated that a significant proportion of the total number of EGF-R molecules were phosphorylated in response to EGF.

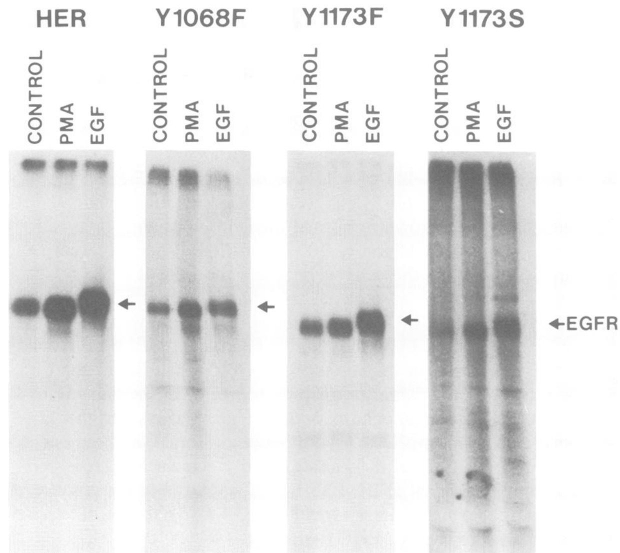

Fig. 5. Phosphorylation of EGF-R in living cells. Cells were incubated for 4 h with [^{32}P]orthophosphate (0.2 mCi/ml) in phosphate-free medium, then either with 10 ng/ml of EGF for 20 min and/or with 100 nM PMA for 30 min at 37°C. Cells were lysed in the presence of phosphatase inhibitors, and the EGF-R was immunoprecipitated with human-specific monoclonal anti-EGF-R antibodies (mAb 108.1) and analyzed on a 5−10% gradient SDS−polyacrylamide gel. In unstimulated cells or in cells stimulated with PMA, phosphate incorporated into the EGF-R was in the form of phosphothreonine and phosphoserine, while in EGF stimulated cells phosphotyrosine, phosphothreonine and phosphoserine were present (Honegger *et al.*, 1987b and data not shown).

A known response of EGF is its capacity to stimulate the phosphorylation of ribosomal protein S6 on serine and threonine residues, probably through activation of a specific S6-kinase (Novak-Hofer, 1984, 1985). S6-phosphorylation is not stimulated by EGF in cells lacking EGF-R or in cells expressing the kinase-negative receptor mutant (data not shown). However, cells expressing either wild-type or mutant receptors incorporated [^{32}P]phosphate into S6 in response to EGF in a similar manner (Figure 7). PMA stimulated S6-phosphorylation in all cell lines irrespective of the presence of an active EGF-R.

EGF is mitogenic for cells expressing mutant receptors

Cells expressing wild-type EGF-R at different expression levels differed in their response to maximally stimulating concentrations of EGF (Figure 8A). The mitogenic response to EGF correlated with the level of expression of the EGF-R in transfected cells: cells expressing a high number of receptors were more responsive than cells expressing low numbers of receptors per cell. At ~500 000 receptors/cell the mitogenic response reached a plateau at a level similar

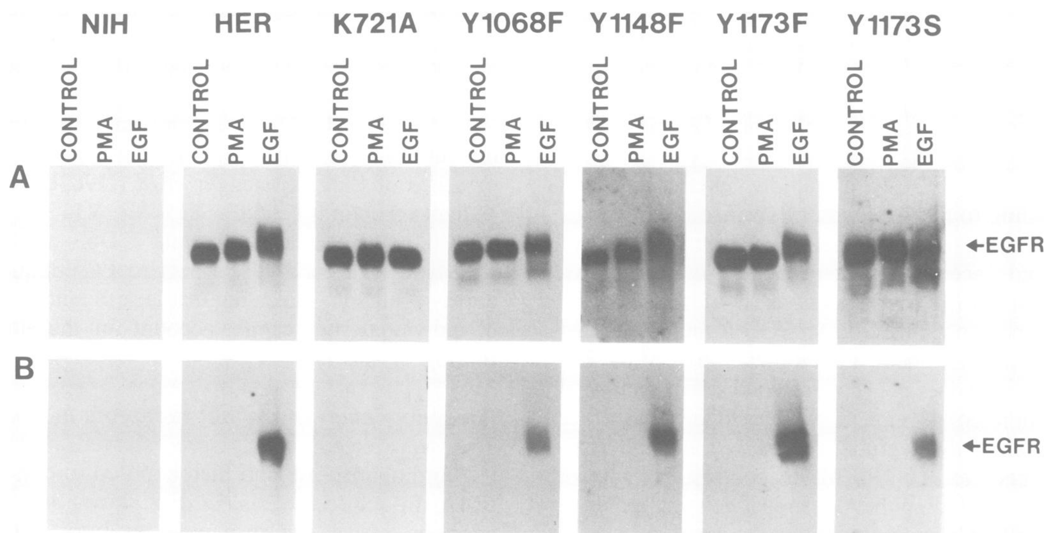

Fig. 6. Tyrosine phosphorylation of EGF-R in living cells. Quiescent cells were stimulated with either 200 ng/ml EGF for 20 min or with 100 nM PMA for 20 min, lysed in the presence of phosphatase inhibitors and immunoprecipitated with monoclonal antibody (mAb 108.1). The sample was divided into two aliquots, analyzed on 7% SDS−PAGE, transferred to nitrocellulose and immunoblotted either with antiserum RK2 against the EGF-R (**A**), or with anti-phosphotyrosine antibodies (**B**). The immunoblots were developed with [^{125}I]protein A, and autoradiographed at room temperature for 3 h (control, HER, K721A, Y1068F and Y1173F) or for 24 h (Y1148F and Y1173S).

to the response induced by 10% fetal calf serum (Figure 8A). In contrast, the concentration of EGF required for half-maximal stimulation of DNA synthesis did not depend on the level of receptor expression.

Transfected cells expressing different levels of mutant receptors with altered autophosphorylation sites showed a similar dependency of the maximal mitogenic response on receptor expression levels (Figure 8B). The maximal response seen in cell lines expressing mutant receptor was similar to the mitogenic response of cell lines expressing comparable numbers of wild-type receptor. However, all cell lines expressing mutant receptors, independent of their receptor expression level, showed a shift of their dose–response curves towards lower concentrations of EGF. The concentration of EGF required for half-maximal stimulation of DNA synthesis was 2–5 times lower in cells expressing mutant receptors than in cells expressing wild-type receptors. In addition, cells expressing mutant receptors consistently showed higher levels of basal [^{3}H]thymidine incorporation in the presence of 0.5% fetal calf serum than cell lines expressing comparable numbers of wild-type receptors (Figure 8C–F).

Discussion

Many of the known protein tyrosine kinases not only phosphorylate exogenous substrates, but also undergo autophosphorylation on multiple endogenous sites. Loss of autophosphorylation sites due to carboxy-terminal deletions seems to be a common lesion in many oncogenes derived from cellular protein tyrosine kinases (reviewed by Yarden and Ullrich, 1988). It was therefore suggested that such deletions may play a role in transformation. While the sequence and structure of the catalytic domain of protein tyrosine family is highly conserved, the number and location of the C-terminal autophosphorylation sites vary between related kinases. Autophosphorylation has been implicated in

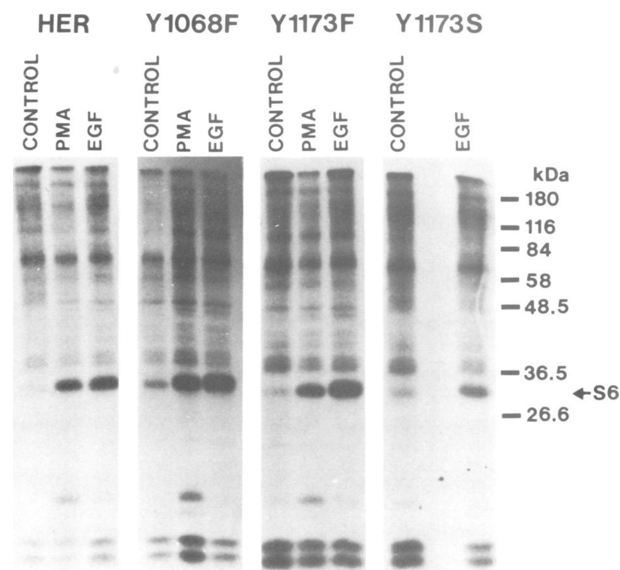

Fig. 7. Stimulation of S6-phosphorylation by EGF and PMA. Ribosomes were isolated from cells labeled with [^{32}P]orthophosphate and stimulated with EGF or PMA as described in the legend to Figure 5. The ribosomal proteins were analyzed on a 12.5% SDS–polyacrylamide gel and autoradiographed; ribosome protein S6 is marked by an arrow.

the regulation of protein tyrosine kinase activity, and the replacement of autophosphorylation site tyrosine residues by phenylalanine has been shown to alter the biological activities of pp$^{60c\text{-}src}$ (Cartwright *et al.*, 1987; Kmiecik and Shalloway, 1987), of the insulin receptor kinase (Ellis *et al.*, 1985) and of pp$^{120gag\text{-}fps}$ (Hansen-Meckling *et al.*, 1987). The main regulatory site in these kinases seems to be a tyrosine residue within the kinase domain, corresponding to tyrosine 845 of the EGF-R.

The three known autophosphorylation sites of EGF-R (Tyr1173, Tyr1148 and Tyr1068) are clustered at the C-terminal end of the EGF-R, downstream of the kinase domain (Downward *et al.*, 1984). While one report provided evidence that autophosphorylation of EGF-R enhances kinase activity (Bertics and Gill, 1985a,b), another report saw no effect of receptor autophosphorylation on the kinetic properties of the EGF-R kinase (Downward *et al.*, 1985).

In this and in the accompanying report (Honegger *et al.*, 1988), we demonstrate that alterations of individual autophosphorylation sites did not have a major effect on the catalytic or biological properties of the mutant receptors. However, a small and reproducible effect was measured for the mitogenic response of the cells expressing mutant receptors, rendering them slightly more sensitive towards EGF. Similarly, autophosphorylation may have a small effect on the apparent affinity of exogenous substrates to the receptor kinase due to the removal of a competitive inhibition by intrinsic substrate sites (Honegger *et al.*, 1988).

Analysis of the properties of a kinase-negative EGF-R mutant K721A indicated that either kinase activity or autophosphorylation determine whether internalized receptor is degraded or returns to the cell surface for re-utilization (Honegger *et al.*, 1987a). Using the K721A mutant alone, it was impossible to determine whether the altered trafficking is a direct consequence of the loss of kinase activity towards exogenous substrates or a consequence of its inability to undergo autophosphorylation. All autophosphorylation site mutants tested in this study behaved like wild-type receptor with respect to receptor internalization, receptor down-regulation and ligand-induced receptor degradation. None of the individual autophosphorylation sites is by itself sufficient to prevent receptor down-regulation, nor are the C-terminal 63 amino acids involved in guiding the internalized receptor into the degradation pathway.

All mutant receptors with altered autophosphorylation sites showed significant autophosphorylation in intact cells, in the range of 50–80% of the phosphorylation of wild-type EGF-R. Although Tyr1173 was thought to be the main *in vivo* autophosphorylation site (Downward *et al.*, 1984), neither Y1173F nor Y1173S showed a greater reduction of *in vivo* autophosphorylation than Y1068F or Y1148F. This suggests that either the loss of the Y1173 site leads to enhanced phosphorylation of the remaining sites or, more likely, that phosphorylation of the three autophosphorylation sites is more evenly distributed than previously suggested (Downward *et al.*, 1984).

The mutant receptors were as potent as wild-type receptor in eliciting responses indirectly linked to the *in vivo* kinase activity of the EGF-R, such as increased phosphorylation of ribosomal protein S6 due to the activation of a S6-kinase. Cells transfected with mutant receptors became mitogenically responsive to EGF, indicating that mutant receptors not only are able to undergo EGF-induced autophosphorylation, but

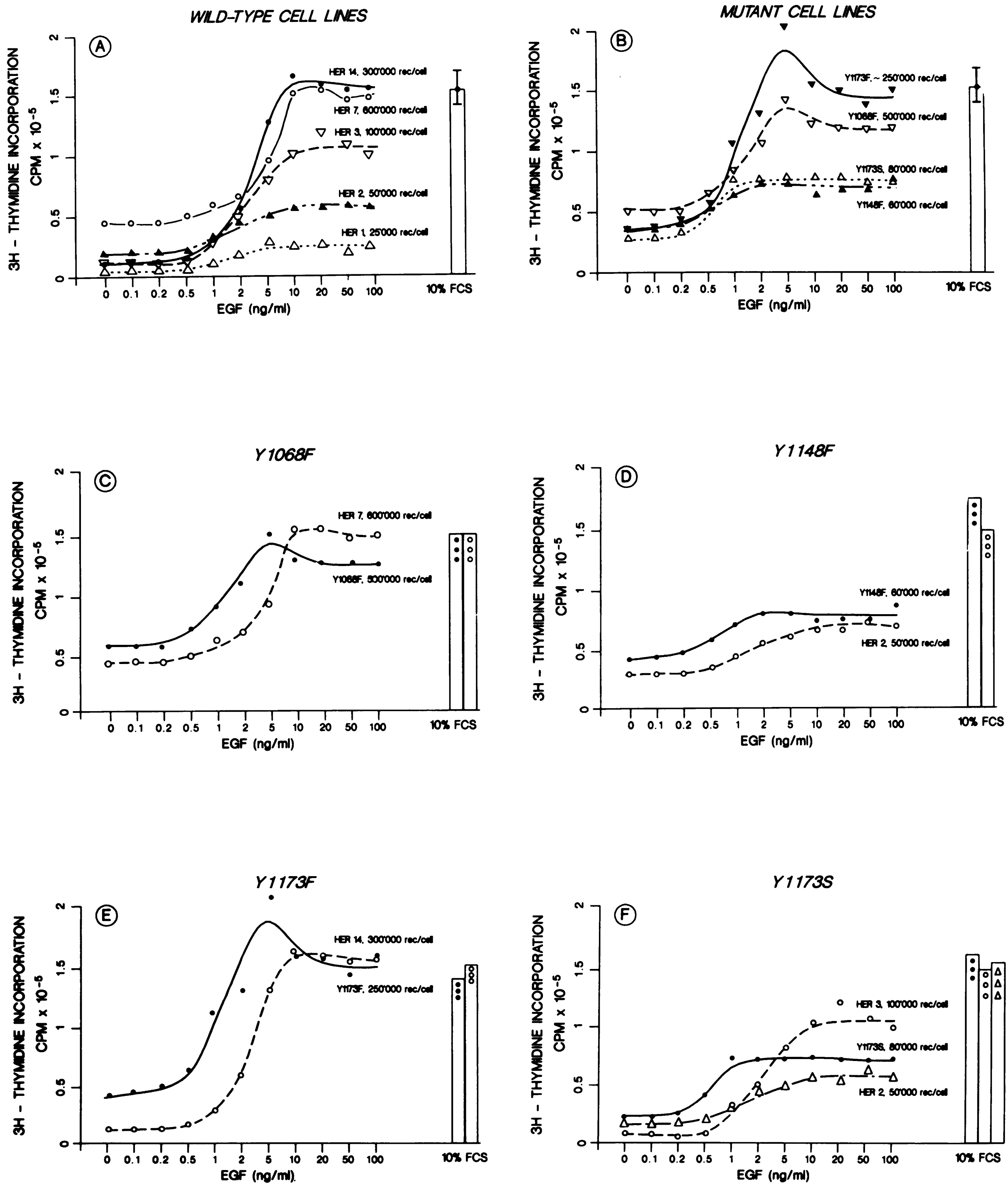

Fig.8. Stimulation of [^{3}H]thymidine incorporation. Cells were starved in 0.5% FCS for 3 days and then stimulated with EGF at different concentrations or with 10% FCS for 18 h before [^{3}H]thymidine was added for 4 h. TCA-insoluble radioactivity was determined and plotted against the EGF dose used for stimulation. (**A**) Cell lines expressing wild-type EGF-R of different expression levels. HER1 (△): 25 000 receptors/cell, half-maximal stimulating dose 2 ng/ml; HER2 (▲): 50 000 receptors/cell, 2 ng/ml; HER3 (▽): 100 000 receptors/cell, 2−5 ng/ml; HER14 (●): 300 000 receptors/cell, 2−5 ng and HER7 (○): 600 000 receptors/cell, 2−5 ng/ml. (**B**) Highest expressing cell line for each type of mutant receptor with altered autophosphorylation site. Y1068F (▽): 500 000 receptors/cell, half-maximal stimulating dose 1 ng/ml; Y1148F (▲): 60 000 receptors/cell, 0.5−1 ng/ml; Y1173F (▼: 200 000 receptors/cell, 1−2 ng/ml and Y1173S (△): 80 000 receptors/cell, 0.5−1 ng/ml.
(**C−F**) Comparison of the responsiveness of mutant and wild-type receptors: each of the cell lines expressing mutant receptor (●) shown in panel B is compared to the most closely matching cell line expressing wild-type receptor (○). Mutant cell lines with lower expression levels than those shown in this figure behaved similarly: each showed a shift of the dose−response curve towards lower concentrations of EGF as compared to cell lines expressing similar amounts of wild-type EGF-R. The magnitude of the response to maximal mitogenic stimulation correlated with the level of receptor expression.

could also recognize and phosphorylate specific cellular substrates which are important for the generation of the mitogenic response. The maximal EGF-induced mitogenic response of cells expressing mutant receptors correlated with receptor expression level in the same way as it did for cells expressing wild-type receptors, indicating that under conditions of maximal stimulation the autophosphorylation site mutants were as active as wild-type receptor. However, the dose–response curves of the autophosphorylation site mutants were consistently shifted to lower ligand concentrations, rendering the cells expressing mutant receptors slightly more sensitive to low concentrations of EGF than the cells expressing similar amounts of wild-type receptors. For cells expressing either wild-type or mutant EGF-R, basal levels of [^{3}H]thymidine incorporation in the presence of 0.5% fetal calf serum showed variability from experiment to experiment. Within the same set of experiments, basal incorporation was elevated in highly overexpressing cells (>500 000 receptors/cell). At lower expression levels, cells expressing mutant receptors with altered autophosphorylation sites showed consistently higher basal incorporation than cells expressing similar amounts of wild-type receptors. Nevertheless, the mutant receptors responded well to stimulation with EGF. Preventing autophosphorylation by replacing any one of the autophosphorylation sites did not abolish the need for ligand activation. Although basal [^{3}H]thymidine incorporation was elevated, the mutant receptors were not constitutively activated.

The EGF binding affinity of the receptor was not affected by the mutations. Receptor occupancy at a given EGF concentration is therefore similar for wild-type and mutant receptors, and the altered responsiveness cannot be explained by altered ligand binding affinities but probably is caused by the effect of the mutations on signal transductions. Autophosphorylation of the EGF-R does not affect the V_{max} of the EGF-R kinase for phosphorylation of peptide substrates, but may lower the K_m by removing an intrinsic competing substrate (Bertics and Gill, 1985a,b; Honegger *et al.*, 1988). Replacing tyrosine by phenylalanine in a substrate peptide representing the sequence flanking the Y1173 site results in decreased competitive inhibition of receptor autophosphorylation compared to the tyrosine-containing peptide. Conversely, it can be assumed that a phenylalanine analog of a receptor autophosphorylation site would be a poorer inhibitor of substrate phosphorylation than an unphosphorylated tyrosine site, thus functionally mimicking a phosphorylated site rather than an unphosphorylated site. If this effect is indeed responsible for the altered sensitivity of the mutant cell lines, the mutation of more than one site should have an additive effect, giving rise to receptor mutants which show higher basal activity and half-maximal stimulation at even lower doses of EGF than single site mutations.

Receptor autophosphorylation may provide a positive feedback mechansm, decreasing the apparent K_m of kinase substrates and thus increasing the kinase activity at low substrate concentration. Mutations which abolish individual autophosphorylation sites may therefore decrease the intrinsic regulatory function provided by autophosphorylation. Whether this mechanism is sufficient to explain the increased sensitivity of the mutant receptors remains to be proved. It is clear that further research is required to explore the validity of this and other potential mechanisms.

Materials and methods

Constructs

In order to obtain the mutants HERY1064F, HERY1148F and HERY1173F, a 564 bp *Sst*II–*Eco*RI fragment and a 110 bp *Bam*HI–*Sst*II fragment were isolated from CHERc and joined in a 3-factor ligation with a *Bam*HI–*Eco*RI fragment of a M13mp18 vector. Single-stranded DNA template was prepared and the mutations were performed using primers 5'-CCAGTGCCTGAATTTATAAACCAGTCCG-3' (Y1068F), 5'-GACAACCCTGACTTTCAGCAGGACTTCT-3' (Y1148F) and 5'-GAAAATGCAGAATTTCTAAGGGTCGCGC-3' (Y1173F). The mutagenesis was based on published procedures (Razin *et al.*, 1978; Hutchinson *et al.*, 1978; Gillam *et al.*, 1979; Gillam and Smith, 1979a,b; Adelman *et al.*, 1983). Briefly, 50 ng of phosphorylated primer DNA was hybridized to 2 mg of ss M13 template. The second strand was synthesized in the presence of nucleotide triphosphates, and closed by ligation. ds circular DNA was transformed into competent *Escherichia coli* JM101 and 18 h after plating plaques were screened as described by Benton and Davis (1977) at high stringency, using the primers as hybridization probes. Mutated sequences were confirmed by M13 dideoxy sequencing (Sanger *et al.*, 1977; Messing *et al.*, 1981) and the replicative form of the mutant M13 vectors was prepared.

To construct the expression plasmids CVN/HERY1068F, CVN/HERY1148F and CVN/HERY1173F, *Bgl*II–*Sst*II fragments of 1067 bp from each of the three mutant M13 rfs were joined with a 10173 bp *Sst*II–*Bgl*II fragment of CVN/HERc. The resulting plasmids were amplified and screened for correctly sized inserts and by hybridization to the primers under highly stringent conditions using the protocol of Benton and Davis (1977).

Transfections

NIH-3T3 cells (clone 2.2) were grown in DMEM (Gibco) with 10% FCS. Cells growing in 10 cm dishes were transfected with 10–20 μg of plasmid DNA/dish using the calcium phosphate precipitation technique (Wigler *et al.*, 1979). Two days after transfection, the cells were split, seeded at a density of 100 000 cells/10 cm dish, and put under neomycin-resistance selection by addition of 0.8 μg/ml of Geneticin G418 (Gibco) to the medium. Resistant clones were picked after 3 weeks and screened for [^{125}I]EGF binding. Positive clones were tested for expression of human EGF-R by immunoprecipitation of [^{35}S]methionine-labeled receptor with human-specific antibodies.

Binding experiments

Binding experiments were performed on confluent cells growing on fibronectin-coated 24-well Costar dishes as previously described (Honegger *et al.*, 1987a). For Scatchard analysis, the cells were incubated for 60 min at room temperature with different concentrations of [^{125}I]EGF 0.03–300 ng/ml; 100 000 c.p.m./ng) in DMEM with 50 mM Hepes and 2 mg/ml BSA. Similar values of nonspecific binding were determined either by addition of excess unlabeled EGF (1–6 μg/ml), or by parallel binding experiments to parental cells which are devoid of EGF-R.

Internalization of [^{125}I]EGF and receptor down-regulation

Cells were incubated with 100 ng/ml [^{125}I]EGF for 90 min at 4°C, then warmed to 37°C for different time periods and excess unbound [^{125}I]EGF removed. Surface associated [^{125}I]EGF was separated from cell associated [^{125}I]EGF molecules by washing the cells with a solution containing 0.5 M acetic acid and 150 mM NaCl. The cells were lysed for 30 min in 1 M NaOH at 37°C, and the surface-associated and cell-associated radioactivities were determined separately (Honegger *et al.*, 1987a).

Biosynthetic half-life of the EGF-R

Partially confluent cell cultures grown on fibronectin-coated 10 cm dishes were labeled overnight with 50 μCi/ml of [^{35}S]methionine in methionine-free DMEM containing 10% calf serum, washed three times with DMEM and incubated for various times up to 32 h in DMEM containing 10% calf serum with or without 200 ng/ml EGF. The cells were lysed and the EGF-R was immunoprecipitated with human-specific anti-EGF-R antibodies (mAb 108.1) as previously described (Honegger *et al.*, 1987a). The immunoprecipitates were analyzed by SDS–PAGE and the intensity of the EGF-R bands was quantitated by densitometric scanning of the autoradiographs.

Phosphorylation experiments

Cells were grown to confluence in 10 cm dishes and starved overnight in 0.5% serum. Then, the cells were washed two times with phosphate-free DMEM and incubated for 4 h with 5 ml of phosphate-free DMEM containing 25 mM Hepes, pH 7.5, and 0.2 mCi/ml of [^{32}P]orthophosphate.

EGF (100 ng/ml) or PMA (100 nM) were added and the cells were incubated for 20 or 30 min respectively at 37°C. The cells were placed on ice and, after a quick rinse with phosphate-free DMEM, lysed in 2 ml of 50 mM Tris, pH 7.0, 250 mM sucrose, 1 mM EGTA, 5 mM dithiothreitol, 1.5 mM $MgCl_2$, 2% (v/v) Triton X-100, 100 mM sodium fluoride, 30 mM *p*-nitrophenylphosphate, 10 mM sodium pyrophosphate, 2 mM sodium orthovanadate, 2 mM PMSF, 1 mg/ml aprotinin and leupeptin. The cells were incubated for 5 min on ice, scraped into centrifuge tubes and centrifuged for 5 min at 7000 *g*. The supernatants were subsequently transferred to a new tube and centrifuged for 1 h at 100 000 *g*. The EGF-R was immunoprecipitated from the high-speed supernatant with protein A–Sepharose anti-EGF-R antibody complex as previously described (Honegger *et al.*, 1987b). The pellet containing the ribosomes was resuspended in 1 ml of lysis buffer and layered on a cushion containing 1.1 M sucrose, 500 mM KCl, 5 mM $MgCl_2$, 20 mM Tris–HCl, pH 7.4, and 6 mM β-mercaptoethanol and centrifuged overnight at 100 000 *g*. The supernatant was discarded and the pellet was resuspended in 500 ml of 100 mM KCl, 2 mM $MgCl_2$, 20 mM Tris–HCl, pH 7.4, 6 mM β-mercaptoethanol. Then, 1 M $MgCl_2$ was added to a final concentration of 10 mM and 0.7 vol of cold ethanol added to precipitate the ribosomes. The precipitate was spun down, lyophilized and extracted twice by resuspending in 150 ml of the same buffer, increasing the $MgCl_2$ concentration to 100 mM, adding 2 vols of glacial acetic acid, incubating for 45 min on ice and centrifugation for 15 min at 7000 *g*. Subsequently, 5 vols of cold acetone (−20°C) were added to the combined supernatants, and incubated overnight at −20°C. The precipitate was spun down, washed twice with cold acetone, lyophilized, resuspended in SDS sample buffer, incubated for 5 min at 95°C and analyzed on a 12.5% SDS–polyacrylamide gel.

Tyrosine phosphorylation of EGF-R in living cells

Cells were grown to confluence in 10 cm dishes, starved overnight in DMEM containing 0.5% calf serum and stimulated for 20 min with 200 ng/ml EGF or for 30 min with 100 nM PMA. The cells were lysed in 0.5 ml of 50 mM Hepes, 150 mM NaCl, 1.5 mM $MgCl_2$, 1 mM EGTA, 10% glycerol, 1% Triton X-100, 1 mM PMSF, 1 mg/ml aprotinin and leupeptin, 100 mM sodium fluoride, 30 mM *p*-nitrophenyl phosphate, 10 mM sodium pyrophosphate and 0.2 mM sodium orthovanadate and centrifuged for 15 min at 10 000 *g*. The EGF-R was immunoprecipitated from the supernatant using monoclonal antibodies against the extracellular domain specific to the human receptor (mAb 108.1). Each sample was divided and analyzed on two 7% SDS–polyacrylamide gels. Each gel was transferred to nitrocellulose for immunoblotting either with antibodies which recognize cytoplasmic epitopes of EGF-R (RK2), or with anti-phosphotyrosine antibodies (gift from P.Comoglio). [^{123}I]Protein A was used to detect the antibodies by autoradiography. Radioactive bands were cut out and counted in a γ-counter to compare the ratio between anti-EGF-R radioactivity and anti-phosphotyrosine radioactivity.

[^{3}H]Thymidine incorporation

Cells were seeded at a density of 100 000 cells/well in fibronectin-coated 24-well Costar dishes and grown for 2 days in 10% calf serum, then starved for 3 days in 0.5% calf serum. EGF or FCS was added and the cells were incubated for 18 h. [^{3}H]Thymidine was added and after 4 h the cells were washed three times with PBS, incubated with ice-cold 5% tricholoroacetic acid for 30 min on ice and washed three times with PBS. The TCA precipitate was solubilized in 0.2 N NaOH for 30 min at 37°C and counted in a scintillation counter.

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Received on February 23, 1988; revised on June 20, 1988