Release of a Phorbol Ester-Induced Mitogenic Block by Mutation at Thr-654 of the Epidermal Growth Factor Receptor

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The tumor promoter phorbol ester (TPA) modulates the binding affinity and the mitogenic capacity of the epidermal growth factor (EGF) receptor. Moreover, TPA-induced kinase C phosphorylation occurs mainly on Thr-654 of the EGF receptor, suggesting that the phosphorylation state of this residue regulates ligand-binding affinity and kinase activity of the EGF receptor. To examine the role of this residue, we prepared a Tyr-654 EGF receptor cDNA construct by in vitro site-directed mutagenesis. Like the wild-type receptor, the mutant receptor exhibited typical high- and low-affinity binding sites when expressed on the surface of NIH 3T3 cells. Moreover, TPA regulated the affinity of both wild-type and mutant receptors and stimulated receptor phosphorylation of serine and threonine residues other than Thr-654. The addition of TPA to NIH 3T3 cells expressing a wild-type human EGF receptor blocked the mitogenic capacity of EGF. However, this inhibition did not occur in cells expressing the Tyr-654 EGF receptor mutant. In the latter cells, EGF was able to stimulate DNA synthesis even in the presence of inhibitory concentrations of TPA. While phosphorylation of sites other than Thr-654 may regulate ligand-binding affinity, the phosphorylation of Thr-654 by kinase C appears to provide a negative control mechanism for EGF-induced mitogenesis in mouse NIH 3T3 fibroblasts.

The mitogenic polypeptide epidermal growth factor (EGF) mediates its biological responses by activating an integral membrane glycoprotein denoted as the EGF receptor (reviewed in references 5 and 26; J. Schlessinger, *Biochemistry*, in press). Scatchard analysis of binding of ¹²⁵I-EGF to intact cells reveals two affinity classes of EGF toward the receptor (16). The addition of the phorbol ester (TPA) to cells abolishes the high-affinity binding sites (4, 16, 18, 27) and reduces the protein-tyrosine kinase activity of the receptor molecule (6, 9, 15). Moreover, TPA-induced kinase C phosphorylation of the EGF receptor occurs mainly on Thr-654 (7, 13), suggesting that the phosphorylation of this residue regulates the ligand-binding affinity and the kinase activity of the EGF receptor. The phosphorylation state of Thr-654 may also play a role in endocytosis as an Ala-654 mutant of the EGF receptor fails to internalize in response to TPA, while the native EGF receptor undergoes TPA-induced internalization (19). We have shown that mutations in the cytoplasmic domain of the EGF receptor can cause the loss of high-affinity EGF-binding sites (20, 23). Hence, the so-called effect of TPA on EGF receptor internalization (2, 19, 26; Schlessinger, in press) most probably represents TPA-induced loss of high-affinity receptors rather than receptor internalization (26; Schlessinger, in press). The physiological role of the high- and low-affinity receptors is not known (26). Interestingly, growth factors other than EGF such as platelet-derived growth factor and bombesin, which bind to their own distinct membrane receptors, are also able to abolish the high-affinity state of the EGF receptor (reviewed in reference 26; Schlessinger, in press). This process, termed receptor transmodulation, is thought to be mediated by kinase C as both platelet-derived growth factor and bombesin activate this enzyme through the activation of phosphoinositide breakdown (reviewed by Schlessinger, in

press). Further insight into the mechanism of EGF receptor transmodulation was obtained from the analysis of the properties of an insertional mutant of the EGF receptor (21, 23). The insertional mutant is displayed on the cell surface with typical high- and low-affinity binding sites. However, unlike wild-type receptors, TPA is unable to modulate the ligand-binding affinity and to stimulate the phosphorylation of the insertional mutant (21). Hence, the phosphorylation of the EGF receptor induced by TPA may be linked to the capacity of TPA to modulate the binding affinity of EGF receptors.

In this report, we explore the role of Thr-654 of the EGF receptor by studying the properties of a Tyr-654 mutant of the EGF receptor. We show that, like the wild-type receptor, the Tyr-654 receptor mutant exhibits both high- and low-affinity binding sites for EGF and that TPA regulates their ligand-binding affinity in a similar manner. Hence, phosphorylation of sites other than Thr-654 appears to play a role in regulation of receptor ligand affinity. However, the phosphorylation state of Thr-654 may provide a negative control mechanism for EGF-induced mitogenesis in murine NIH 3T3 cells.

MATERIALS AND METHODS

Construction of Tyr-654 EGF receptor mutant. To obtain the Tyr-654 mutant, we joined a 2,885-base-pair (bp) *HindIII*-*BglII* fragment and a 134-bp *BglII*-*EcoRI* fragment of CVS HERC (24) to a *HindIII*-*EcoRI* fragment of M13mp10 in a three-factor ligation. Single-stranded template was prepared for site-directed mutagenesis with a primer 5'-CGGAGCGCTACCTGCGGAGG-3'. The mutagenesis was based on published protocols (1, 10-12). Phosphorylated primer DNA (50 ng) was hybridized to 2 µg of single-stranded M13 template. The second strand was synthesized in the presence of nucleotide triphosphates and closed by ligation. Double-stranded circular DNA was transformed into competent *Escherichia coli* JM101, and 18 h after plating, plaques were screened as described by Benton and

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Davis (3) at high stringency with the primer as a hybridization probe. The mutated sequence was confirmed by M13 dideoxy sequencing (22, 25), and the replicative form of the mutant M13 vector was prepared.

To construct the expression plasmid CVS/HER T654Y, we joined a 2,885-bp *HindIII*-*BglIII* fragment from the mutant M13 replicative form in a three-factor ligation with 5,288-bp *BglIII*-*PvuI* and 1,163-bp *PvuI*-*HindIII* fragments from CVS/HERc (21). The resulting plasmids were screened for correctly sized inserts and by hybridization to the primer under highly stringent conditions by the protocol of Benton and Davis (3).

Transfections. NIH 3T3 cells were grown in Dulbecco modified Eagle medium supplemented with 10% fetal calf serum. Transfections were by the calcium phosphate precipitate technique. Cloned DNA (10 μ g) was used to transfect a 3.5-cm plate seeded with 5×10^5 cells 24 h before transfection. At 2 to 3 days later, the cells were split (1:20) and put under selection with the antibiotic Geneticin (G418), 0.8 mg/ml (GIBCO Laboratories, Grand Island, N.Y.).

Antibodies. The following antibodies specific for the EGF receptor were used. RK-2 is a rabbit antipeptide antiserum previously described (17). Anti-C terminus is a rabbit antiserum raised against the C-terminal 13-amino-acid peptide of the human EGF receptor.

Immunoprecipitation experiments. Subconfluent NIH 3T3 cells expressing the wild-type receptor (pLNT1-3 cells) or the Tyr-654 EGF receptor mutant (pLNT654Y cells) were labeled with [35 S]methionine and cysteine (50 Ci/ml; Amersham Corp., Arlington Heights, Ill.) in methionine- and cysteine-free Dulbecco-modified Eagle medium containing 2% dialyzed fetal calf serum for 4 to 5 h or overnight. Then the labeled cells were washed with phosphate-buffered saline and solubilized in 0.5 ml of solubilization buffer containing 20 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid) (pH 7.5), 150 mM NaCl, 10% glycerol, 1% Triton, 1.5 mM $MgCl_2$, 1.0 mM EGTA, 1% aprotinin, and 1 mM phenylmethylsulfonyl fluoride. The solubilized cells were centrifuged for 10 min at 4°C, and the resulting supernatant was incubated with anti-EGF receptor antibodies bound to Sepharose-protein A (Pharmacia, Inc., Piscataway, N.J.) for 30 to 120 min at 4°C. Immunoprecipitates were washed three times with 50 mM HEPES (pH 8.0)–500 mM NaCl–0.1% sodium dodecyl sulfate–0.2% Triton X-100–5 mM EGTA and three additional times with the same buffer containing 150 mM NaCl. Sample buffer was added to washed immunoprecipitates and heated for 3 min at 95°C. The samples were analyzed by gel electrophoresis on a 5 to 10% polyacrylamide gel containing sodium dodecyl sulfate. Gels were treated with AMPLIFY (Amersham) for 30 min, dried, and exposed for 10 h at –70°C for autoradiography on Kodak XAR-5 film.

125 I-EGF binding experiments. 125 I-EGF was prepared by the lactoperoxidase method, and mouse EGF was obtained from I.D.L. (Jerusalem). Cells expressing wild-type human EGF receptor and its mutant were seeded in 24-well Costar dishes at a density of 5×10^4 cells per well 2 days before the binding experiments were done. Cells were washed twice with Dulbecco modified Eagle medium containing 0.1% bovine serum albumin buffered with 20 mM HEPES (pH 7.5) and then incubated with various concentrations of 125 I-EGF in the same medium either for 2 h at 4°C or for 1.5 h at room temperature. Then the cells were washed twice with medium and solubilized with 1 ml of 0.2 N NaOH. After 60 min at 37°C, the radioactivity of the solubilized cells was determined. To determine the effect of TPA on the binding of

125 I-EGF, we incubated the cells for 1 h at 37°C with the same buffer containing 10 nM TPA. Subsequently, 125 I-EGF binding was done as described above. Similar results were obtained when 10 nM of other analogs of TPA or 100 nM TPA was used. Dimethyl sulfoxide did not have any effect on the binding of 125 I-EGF. Nonspecific binding was determined by parallel incubation of each concentration of 125 I-EGF with excess (100 times) unlabeled EGF. All incubations were performed in duplicate. Specific binding was calculated as total cell-associated radioactivity minus nonspecific cell-associated radioactivity. Nonspecific binding did not exceed 10% of the total bound radioactivity. Similar results were obtained in three separate experiments. The number of receptors per cell and the K_d for their binding were determined from Scatchard plots by using a computer program developed by S. Felder. Scatchard plots represent the least-square analysis best fit of either two or a single affinity class.

Phosphorylation experiments. Subconfluent cells were washed with 5 ml of phosphate-free medium and incubated for 14 h with 1 mCi of $^{32}P_i$ per ml in 3 ml of medium containing 1% dialyzed fetal calf serum. TPA (100 nM) was added for the last 30 min of labeling before lysis. Cells were solubilized in solubilization buffer containing 20 mM HEPES (pH 7.5), of 150 mM NaCl, 10% glycerol, 1% Triton X-100, 1.5 mM $MgCl_2$, 1 mM EGTA, 10 μ g of aprotinin and leupeptin per ml, 200 μ M sodium vanadate, and 20 mM sodium fluoride. The EGF receptor was immunoprecipitated from the cell lysate with RK-2 antibody as previously described (17). Similar results were observed when other anti-EGF receptor antibodies were used for the immunoprecipitation experiments. Two-dimensional phosphoamino acid analysis of the EGF receptor was done by the procedure of Hunter and Sefton (14). Analysis of tryptic phosphopeptides was performed on 100- μ m cellulose thin-layer plates. First-dimension electrophoresis was performed for 30 min at pH 8.9 with 1,000 V. The second-dimension chromatography was done by published procedures (14).

DNA synthesis. Cells were grown to confluence for 2 to 3 days in 24-well Costar dishes. The medium was changed to Dulbecco modified Eagle medium containing 1% fetal calf serum and then incubated for 18 h in the presence or absence of EGF (5 or 25 ng/ml) or with TPA (10 nM) or with both EGF and TPA as indicated. A similar level of thymidine incorporation was obtained with either 5 or 25 ng/ml of EGF. [3 H]thymidine (5 μ Ci per well) was added for 4 h at 37°C. Cells were washed twice with ice-cold phosphate-buffered saline, and then 5% trichloroacetic acid was added for 30 min on ice. Wells were then washed twice with phosphate-buffered saline and solubilized with 0.2 N NaOH, and the radioactivity was determined. Each bar in the figures represents the average of triplicate values.

RESULTS AND DISCUSSION

To examine the possible regulatory role of Thr-654, we used in vitro site-directed mutagenesis to replace Thr-654 of the human EGF receptor with a tyrosine residue (Fig. 1), a potential phosphate acceptor site which cannot be phosphorylated by kinase C. The Tyr-654 EGF receptor cDNA construct was cloned into an expression plasmid containing the simian virus 40 early promoter and the neomycin resistance gene (16). The plasmid was transfected into NIH 3T3 cells, and G418 was used for the selection of cells expressing the receptor mutant. Several cloned cell lines expressing the wild-type EGF receptor or the Tyr-654 receptor mutant were developed. All key experiments presented in this study were

FIG. 1. Structure of Tyr-654 EGF receptor mutant. The cDNA clone of the Tyr-654 mutant was constructed as described in Materials and Methods. The point mutation was made by changing three nucleotide bases of codon 654, using a synthetic oligonucleotide. S, Signal peptide; TM, transmembrane region; tyr, tyrosine.

repeated with two to three cell lines expressing the two constructs with essentially the same results. The NIH 3T3 cells used for transfection experiments were previously characterized and were shown to express undetectable amounts of endogenous murine EGF receptors (21). The transfected cells were grown, and various antibodies against EGF receptor were used for the immunoprecipitation of the human EGF receptor and its mutant. Some of the antibodies used recognize specifically the human EGF receptor. Figure 2 depicts sodium dodecyl sulfate-polyacrylamide gel electrophoretic analysis of biosynthetically labeled cells immunoprecipitated with an antibody (RK-2) directed against a synthetic peptide from the human EGF receptor (17). It appears that both cell lines express polypeptides of 170,000 molecular weight, while the control untransfected cells do not express this polypeptide (Fig. 2). A polypeptide of 170,000 molecular weight was also detected by a standard immunoprecipitation-autophosphorylation assay, indicating that like the wild-type EGF receptor (20, 21, 23), the Tyr-654 EGF receptor mutant is also able to undergo EGF-induced autophosphorylation (see Fig. 4B). Hence, the transfected cells express a functional EGF receptor of appropriate size and enzymatic properties.

Analysis of ^{125}I -EGF binding experiments by the Scatchard method revealed nonlinear curves for the wild-type EGF receptor and for the Tyr-654 EGF receptor mutant which

FIG. 2. Immunoprecipitation of biosynthetically labeled EGF receptors. NIH 3T3 cells expressing wild-type EGF receptor (pLN1-3), NIH 3T3 cells expressing the Tyr-654 EGF receptor mutant (pLNT654Y), or control parental NIH 3T3 cells were labeled with [^{35}S]methionine and subjected to immunoprecipitation with polyclonal antibodies against EGF receptor RK-2 as described in Materials and Methods. kDa, Kilodaltons.

were interpreted as an indication for two affinity (13) binding sites (Fig. 3). The cell lines expressing wild-type EGF receptors (pLN1-3 cells) possess 435,000 receptors per cell of which 5% are high-affinity receptors ($K_d \approx 2.8 \times 10^{-10} \text{ M}$) and the remaining are low-affinity receptors ($K_d \approx 4.7 \times 10^{-9} \text{ M}$). The cells expressing the Tyr-654 EGF receptor mutant (pLNT654Y cells) possess 313,000 receptors per cell of which 8% are high-affinity receptors ($K_d \approx 5.3 \times 10^{-10} \text{ M}$) and 92% are low-affinity receptors ($K_d \approx 9 \times 10^{-9} \text{ M}$) (Fig. 3). The addition of 10 nM TPA for 1 h at 37°C abolished the high-affinity binding of both receptors, reducing their K_d for EGF to $\sim 1 \times 10^{-8} \text{ M}$ (Fig. 3; Table 1). A similar loss of high-affinity binding sites by TPA treatment was also observed in Chinese hamster ovary cells (CHO cells) lacking endogenous EGF receptors which were transfected by either wild-type EGF receptor or Tyr-654 EGF receptor mutant (Table 2). Clearly, TPA can regulate the affinity of both the wild-type EGF receptor and the Tyr-654 EGF receptor mutant in a similar manner (Tables 1 and 2). Similar results were obtained with the TPA analogs 1-oleoyl-2-ocetyl-racglycerol (25 $\mu\text{g/ml}$) and 1,2-dicapryloxy-racglycerol (10 $\mu\text{g/ml}$). Dimethyl sulfoxide did not have any effect.

A TPA-induced loss of high-affinity binding sites of wild-type and mutant EGF receptors was also observed when the

FIG. 3. Effect of TPA on the binding of ^{125}I -EGF to NIH 3T3 cells expressing either wild-type EGF receptors (pLN1-3) or Tyr-654 EGF receptor mutant (pLNT654Y). Transfected cells were treated with 10 nM TPA for 2 h before measurement of ^{125}I -EGF binding at 23°C , and the binding data were analyzed by the Scatchard method as described in Materials and Methods. Inserts show ^{125}I -EGF binding curves in the absence ($\circ$) or presence ($\bullet$) of TPA. A similar effect of TPA was obtained when the binding of ^{125}I -EGF was performed at 4°C (Table 1). This experiment was repeated three times with essentially the same results.

TABLE 1. ^{125}I -EGF binding to transfected NIH 3T3 cells: receptor number and ligand affinity^a

Cell line	Receptor no./cell (10^4)	Affinity, K_d (M)
Room temperature		
pLN1-3	2.5 ± 0.2	$(2.8 \pm 1) \times 10^{-10}$
	41 ± 1.1	$(4.7 \pm 1) \times 10^{-9}$
pLN1-3 + TPA	40.0 ± 1.1	$(9.5 \pm 2) \times 10^{-9}$
pLNT654Y	2.3 ± 0.1	$(5.3 \pm 0.1) \times 10^{-10}$
	29.2 ± 0.2	$(9 \pm 0.2) \times 10^{-9}$
pLNT654Y + TPA	25 ± 0.3	$(1.1 \pm 0.1) \times 10^{-8}$
4°C		
pLN1-3	2.2 ± 0.2	$(1.5 \pm 0.3) \times 10^{-11}$
	39 ± 0.1	$(5 \pm 0.1) \times 10^{-9}$
pLN1-3 + TPA	36 ± 0.3	$(3 \pm 0.15) \times 10^{-9}$
pLNT654Y	1.5 ± 0.1	$(4.3 \pm 0.2) \times 10^{-11}$
	18.8 ± 0.1	$(3.2 \pm 0.1) \times 10^{-9}$
pLNT654 + TPA	18.2 ± 0.2	$(3 \pm 0.1) \times 10^{-9}$

^a The number of receptors per cell for each cell line and the K_d of their binding were determined from Scatchard plots as presented in Fig. 3. The results are the average of $\pm$ standard deviation of three experiments, each done in duplicate.

^{125}I -EGF binding experiments were performed at 4°C (Table 1). At this temperature, the internalization of ^{125}I -EGF into the cells is minimal (5, 26). The K_d of both high- and low-affinity binding sites measured at 4°C is different from the values determined by measurements performed at room temperature. The molecular nature of the high- and low-affinity binding sites of the EGF receptor is not understood. Lin et al. (19) described the expression of an Ala-654 EGF receptor mutant in rat-1 cells. One cell line displayed on its cell surface approximately 170,000 essentially low-affinity EGF receptors ($K_d = 1.9 \times 10^{-9}$ M and $K_d = 4.16 \times 10^{-9}$ M). In these cells, a typical high-affinity state was not detected and TPA did not affect the binding affinity of the mutant receptors. Another cell line expressed 35,000 EGF receptors per cell of which approximately 16,000 receptors were endogenous rat EGF receptors. It was suggested (19) that TPA affects exclusively the binding affinity of the endogenous receptor. However, it is not clear how the specific contribution of the endogenous and mutant receptors for the total binding were determined. A third transfected cell line (B82) expressed the Ala-654 mutant receptor with a single binding affinity which was not reduced by TPA treatment. This result was difficult to interpret because the normal Thr-654 EGF receptor counterpart could not be expressed in these cells.

To analyze further the effect of TPA on EGF receptor function, we examined its influence on the phosphorylation

TABLE 2. ^{125}I -EGF binding to transfected CHO cells: receptor number and ligand affinity^a

Cell line	Receptor no./cell (10^4)	Affinity, K_d (M)
pLCH	0.5 ± 0.2	$(3 \pm 0.6) \times 10^{-10}$
	4.4 ± 0.5	$(3.8 \pm 1.6) \times 10^{-9}$
pLCH + TPA	4.6 ± 0.5	$(1.5 \pm 0.5) \times 10^{-8}$
pLCHT654Y	0.5 ± 0.03	$(6 \pm 0.6) \times 10^{-10}$
	25.7 ± 0.1	$(6 \pm 2) \times 10^{-9}$
pLCHT654Y + TPA	28.2 ± 1	$(3.2 \pm 0.2) \times 10^{-8}$

^a pLCH cells are CHO cells lacking endogenous EGF receptors and expressing wild-type receptors, and pLCHT654Y cells are CHO cells expressing the Tyr-654 EGF receptor mutant. The binding experiments were performed at room temperature, and the binding parameters were determined from Scatchard plots as shown in Fig. 3.

FIG. 4. Effects of EGF or TPA on the phosphorylation of either wild-type EGF receptor (pLN1-3) or Tyr-654 EGF-receptor mutant (pLNT654Y). (A) Living subconfluent cells were labeled with ^{32}P as described in Materials and Methods. Subsequently, the cells were treated for 30 min with EGF or TPA or a control with buffer alone. The cells were then solubilized, and the EGF receptor was subjected to immunoprecipitation with the RK-2 antibodies and then separated by 7.5% polyacrylamide gel electrophoresis. The dried gel was autoradiographed with an enhancing screen at -70°C . (B) Solubilized EGF receptors. Lysates from A431 cells (lane 1), untreated cells (lanes 2 and 4), and TPA (10 nM, 2 h)-treated cells (lanes 3 and 5) were immunoprecipitated with anti-EGF receptor antibodies (RK-2). EGF was added to the cell extract before immunoprecipitation. Immunoprecipitates were incubated with $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ as described previously (20). kDa, Kilodaltons.

of EGF receptors in living cells (Fig. 4). Phosphorylation of either wild-type or Tyr-654 mutated receptors was elevated when cells expressing these receptors were treated with EGF or TPA (Fig. 4). Acid hydrolysis of the isolated phosphorylated receptors revealed that TPA stimulated the phosphorylation of both the wild-type EGF receptor and the

FIG. 5. Two-dimensional phosphoamino acid analysis of EGF receptor of TPA-treated cells expressing either wild-type EGF receptors (pLN1-3) or the Tyr-654 receptor mutant (pLNT654Y). The locations of the phosphoamino acid markers are indicated. (a) Phosphoamino acid analysis of the EGF receptor from untreated pLN1-3 cells; (b) phosphorylated EGF receptors from pLN1-3 cells treated with TPA; (c) phosphoamino acid analysis of the EGF receptors from untreated pLNT654Y cells; (d) phosphorylated EGF receptors from pLNT654Y cells treated with TPA. Phosphoamino acid maps were exposed for 4 days at -70°C with an enhancing screen.

Tyr-654 EGF receptor mutant on both threonine and serine residues (Fig. 5). Serine phosphorylation was more pronounced on the Tyr-654 EGF receptor mutant than on the wild-type EGF receptor. No tyrosine phosphorylation was detected in either the wild type or the Tyr-654 EGF receptor mutant in response to TPA (Fig. 5).

FIG. 6. Tryptic phosphopeptide maps of EGF receptors immunoprecipitated from cells expressing either wild-type EGF receptors (pLN1-3) or the Tyr-654 EGF receptor mutants (pLNT654Y). Subconfluent cells were labeled with $^{32}\text{PO}_4$, and the EGF receptor was immunoprecipitated with RK-2 antibodies and analyzed by polyacrylamide gel electrophoresis. The phosphopeptides obtained from trypsin digestion of the EGF receptors were resolved by electrophoresis at pH 8.9 (anode at left) followed by ascending chromatography on 100- μm cellulose-coated thin-layer plates. (a) Untreated control NIH 3T3 cells expressing the wild-type receptor (pLN1-3). (b) pLN1-3 cells treated with 10 nM EGF for 30 min. (c) pLN1-3 cells treated with 100 nM TPA for 30 min. (d) Untreated control NIH 3T3 cells expressing the Tyr-654 EGF receptor mutant (pLNT654Y). (e) pLNT654Y cells treated with 10 nM EGF for 30 min. (f) pLNT654Y cells treated with 100 nM TPA for 30 min. Autoradiography was for 3 days with a fluorescent screen.

Comparison of two-dimensional tryptic phosphopeptide maps of TPA-induced phosphorylated wild-type or mutant EGF receptors indicated that three phosphopeptides which are observed in the map of the wild-type receptor are missing from the map of the phosphorylated mutant receptor (Fig. 6). These phosphopeptides migrate in positions similar to those of the phosphopeptides previously assigned as peptides containing Thr-654 (13). Hence, the mutant EGF receptor is phosphorylated in response to TPA on sites in addition to Thr-654 (Fig. 4 to 6) (7) which are not yet identified. The addition of TPA to both cell lines also reduced the capacity of both the wild type and the Tyr-654 EGF receptor mutant to undergo autophosphorylation (Fig. 4B) as previously described for the EGF receptor of A431 cells (6, 7). This suggests that TPA was able to reduce the intrinsic kinase activity of wild-type and mutant receptors in a similar manner.

The mitogenic capacity of EGF can be either enhanced or inhibited by TPA depending on cell type (8). In NIH 3T3 cells expressing the wild-type receptor, TPA blocks the

FIG. 7. Effect of TPA on EGF-induced mitogenesis of cell lines expressing either wild-type EGF receptors (pLN1-3) or the Tyr-654 EGF receptor mutants (pLNT654Y). ^3H thymidine incorporation was determined as described in Materials and Methods. Cells were incubated in the presence or absence of EGF or TPA for 18 h before ^3H thymidine addition. Maximal response was obtained with 25 ng of EGF per ml. ^3H thymidine was added for 4 h, and trichloroacetic acid-precipitable radioactivity was determined. The values are the mean of triplicate experiments, and the error bars indicate the standard deviation.

mitogenic capacity of EGF (Fig. 7). The same results were obtained with various concentrations of EGF (5 to 50 ng/ml). Moreover, both cell lines exhibited a similar dose-response curve for EGF-induced mitogenesis with maximal response at 25 and 50 ng of EGF per ml for cells expressing either wild-type or mutant EGF receptors. Similar results were obtained in other transfected NIH 3T3 and CHO cells.

Interestingly, the mitogenic block induced by TPA in cells expressing wild-type EGF receptor did not occur in the cells expressing the Tyr-654 EGF receptor mutant. In these cells EGF was able to stimulate DNA synthesis even in the presence of 100 nM TPA. Thus, mutation at Thr-654 appears to release a TPA-induced mitogenic block conferred on the wild-type EGF receptor. This experiment was repeated with two additional NIH 3T3 and CHO cell lines expressing the Tyr-654 receptor mutant with essentially the same results. On the basis of these results, we propose that kinase C phosphorylation of Thr-654 provides a negative control mechanism for the regulation of the mitogenic response of cells to EGF. It was reported that in A431 cells EGF induces the phosphorylation of Thr-654 of the EGF receptor probably by activation of kinase C, potentially leading to a negative-feedback reaction to EGF action (28). This information together with the results presented here may provide an explanation for the well-documented inhibitory effect of EGF on A431 cell proliferation.

According to our results, TPA regulates the affinity of the Tyr-654 EGF receptor mutant in a way similar to the manner in which it regulates the affinity of the wild-type and native EGF receptors. Phosphoamino acid analysis of the Tyr-654 EGF receptor mutant showed that serine and threonine are the only amino acid residues to be phosphorylated in response to TPA, which suggests that TPA induced the phosphorylation of sites on the EGF receptor in addition to Thr-654 (Fig. 5) (7). The phosphorylation of these additional unidentified serine and threonine residues may regulate the ligand-binding affinity and the protein-tyrosine kinase activity of the EGF receptor. However, the phosphorylation of Thr-654 of the EGF receptor probably manifests a more subtle change which does not influence the binding properties of the EGF receptor expressed in the NIH 3T3 cells, but does have an important allosteric effect on its capacity to stimulate DNA synthesis once activated by EGF.

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