Multisite Phosphorylation of the Epidermal Growth Factor Receptor

USE OF SITE-DIRECTED MUTAGENESIS TO EXAMINE THE ROLE OF SERINE/THREONINE PHOSPHORYLATION*

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The major sites of serine and threonine phosphorylation of the human epidermal growth factor (EGF) receptor observed in intact cells are Thr<sup>654</sup>, Thr<sup>664</sup>, Ser<sup>610</sup>, and Ser<sup>517</sup>. Phosphorylation of the EGF receptor is increased at these sites in cells treated with platelet-derived growth factor or phorbol ester. This increase in EGF receptor phosphorylation is associated with an inhibition of the high affinity binding of EGF to cell surface receptors and an inhibition of the receptor tyrosine protein kinase activity. In order to test the hypothesis that the phosphorylation of the EGF receptor is mechanistically related to the modulation of EGF receptor function, we replaced the major sites of serine and threonine phosphorylation with alanine residues. EGF receptors containing single point mutations or multiple mutations were expressed in Chinese hamster ovary cells.

Analysis of the regulation of the EGF receptor tyrosine protein kinase activity demonstrated that phorbol ester caused an inhibition of the tyrosine phosphorylation of wild-type receptors and receptors lacking Thr<sup>664</sup>, Ser<sup>610</sup>, or Ser<sup>517</sup>. In contrast, the inhibition of EGF receptor tyrosine phosphorylation caused by phorbol ester was not observed for any of the mutated EGF receptors that lacked Thr<sup>654</sup>. These data are consistent with the hypothesis that the phosphorylation of the EGF receptor at Thr<sup>654</sup> is required for the inhibition of the receptor tyrosine protein kinase activity caused by phorbol ester. Investigation of the apparent affinity of the EGF receptor demonstrated that treatment with phorbol ester caused an inhibition of the high affinity binding of <sup>125</sup>I-EGF to cells expressing wild-type EGF receptors and each of the mutated EGF receptors examined. We conclude that the regulation of the apparent affinity of the EGF receptor is independent of the major sites of serine and threonine phosphorylation of the EGF receptor.

The EGF<sup>1</sup> receptor is a 170-kDa transmembrane glycoprotein. The binding of EGF to the extracellular ligand binding domain of the receptor causes an increase in the tyrosine protein kinase activity of the receptor cytoplasmic domain (1). Incubation of cells with phorbol ester or with PDGF causes a decrease in the high affinity binding of <sup>125</sup>I-EGF to cell surface receptors, a decrease in the receptor tyrosine protein kinase activity, and a marked increase in the state of phosphorylation of the EGF receptor at serine and threonine residues (reviewed by Schlessinger, Ref. 2). The apparent association of receptor phosphorylation with the modulation of receptor activity has been used as evidence for the hypothesis that receptor phosphorylation is mechanistically related to receptor regulation (3).

One site of phosphorylation of the EGF receptor, Thr<sup>654</sup>, is a substrate for protein kinase C (4, 5). The phosphorylation of Thr<sup>654</sup> has been observed after the treatment of WI-38 human fetal lung fibroblasts with phorbol ester (5) and with PDGF (6, 7). In order to establish the role of Thr<sup>654</sup> phosphorylation during protein kinase C regulation of high affinity EGF binding, the effect of site-directed mutagenesis of Thr<sup>654</sup> has been investigated. Lin et al. (8) have reported that the inhibition of EGF binding caused by phorbol ester was not observed in cells expressing EGF receptors that lack Thr<sup>654</sup>. This result suggested that phosphorylation of Thr<sup>654</sup> was required for the modulation of EGF binding caused by phorbol ester (8). However, subsequent studies demonstrated that the replacement of threonine-654 with either an alanine or a tyrosine residue caused no significant change in the regulation of the apparent affinity of the EGF receptor observed after treatment of cells with phorbol ester (9, 10) or with PDGF (11). Together, these data demonstrate that the mechanism of regulation of the high affinity binding of EGF to cell surface receptors is independent of the phosphorylation of the EGF receptor at Thr<sup>654</sup> (9–11).

Protein kinase C causes an inhibition of the tyrosine protein kinase activity of the EGF receptor (12, 13). To test the hypothesis that the phosphorylation of Thr<sup>654</sup> is required for the inhibition of the receptor tyrosine protein kinase, we have examined the effect of mutagenesis of Thr<sup>654</sup> (9). Phorbol ester treatment of cells expressing [Ala<sup>654</sup>]<sub>EGF</sub> receptors did not cause an inhibition of EGF receptor tyrosine protein kinase activity (9). This lack of regulation of the [Ala<sup>654</sup>]<sub>EGF</sub> receptor suggests that the phosphorylation of Thr<sup>654</sup> may be a critical event for the regulation by protein kinase C (9). Strong support for this hypothesis is provided by in vitro reconstitution experiments in which the phosphorylation of Thr<sup>654</sup> by protein kinase C is associated with an inhibition of the receptor tyrosine protein kinase activity (9, 12, 15). However, the contribution of Thr<sup>654</sup> phosphorylation to the overall modulation of receptor function has not been resolved because evidence has been presented indicating that the EGF receptor tyrosine protein kinase activity can be regulated by mechanisms that are independent of Thr<sup>654</sup> phosphorylation (10, 16).
Examination of the role of the phosphorylation of the EGF receptor at Thr\(^{654}\) demonstrates that phosphorylation at this site alone is not sufficient to account for the transmodulation of the EGF receptor after treatment of cells with phorbol ester or PDGF (11). Although Thr\(^{654}\) is the only site of phosphorylation on the EGF receptor catalyzed by protein kinase C in vitro (4, 5, 14), previous studies have demonstrated that protein kinase C activation in vivo results in the phosphorylation of the EGF receptor at several additional sites (5, 17-19). The observation that the phosphorylation state of a mutated [Ala\(^{654}\)]EGF receptor was markedly increased after treatment with phorbol ester suggests that the phosphorylation of these serine and threonine residues may be related to the mechanism of EGF receptor transmodulation (9). The purpose of the study reported here was to investigate the role of the multiple sites of serine and threonine phosphorylation of the EGF receptor. In order for phosphorylation to serve a regulatory function, it is likely that a significant fraction of the EGF receptors will be phosphorylated at the sites that are critical for receptor modulation. Previous studies have demonstrated that the stoichiometry of the phosphorylation of the EGF receptor at many sites is extremely low (7). In addition, there are several sites with a measured stoichiometry of phosphorylation between 0.1 and 0.9 mol of phosphate per mol of receptor in phorbol ester and PDGF-treated cells (7). These major sites of phosphorylation represent a potential mechanism of regulation of the EGF receptor function. To test this hypothesis, we examined the effect of mutagenesis of the major sites of EGF receptor phosphorylation. EGF receptors lacking individual phosphorylation sites as well as receptors lacking multiple phosphorylation sites were expressed in CHO cells. The properties of the wild-type and mutated EGF receptors were investigated in detail. We report that the mechanism of regulation of the apparent affinity of the EGF receptor is independent of the major sites of serine and threonine phosphorylation of the receptor. However, the data obtained strongly support the hypothesis that receptor phosphorylation is an important mechanism of regulation of the EGF receptor tyrosine protein kinase activity.

**EXPERIMENTAL PROCEDURES**

**Materials**

[\(^{7-}\)P]ATP, [\(^{35}\)S]methionine, [\(^{32}\)S]dATP, and Na\(^{32}\)PO\(_4\)) were obtained from Amersham. [H]Thymidine was from ICN. [\(^{32}\)P]Phosphate and [\(^{125}\)I]-goat anti-mouse antibody were obtained from Du Pont-New England Nuclear. EGF was purified (20, 21) and iodinated to a specific activity of 70-90 Ci/g as described (22). Restriction enzymes were from Boehringer. Porcine PDGF was obtained from Boehringer. Amniogentolin and PMA were from Sigma. The monoclonal antiphosphotyrosine antibody (FY20) was obtained from ICN. Sequenase was obtained from United States Biochemical Corp.

**Plasmid Construction**

Oligonucleotide-directed mutagenesis of Thr\(^{654}\) (ACC), Thr\(^{669}\) (ACA), Ser\(^{657}\) (AGC), and Ser\(^{657}\) (TCA) to alanine (CCA, CCG, or CCC) was carried out using 17-mer oligonucleotides according to Zoller and Smith (23) using methods described previously (24). Mutations were confirmed by sequencing using [\(^{35}\)S]dATP, ddTTPs, and Sequenase (25). The wild-type and mutated EGF receptor cDNAs were cloned as 4-kilobase XbaI-HindI1 fragments into the plasmid pX(11). This expression vector contains the murine dihydrofolate reductase gene as a selectable marker and allows the expression of the EGF receptor cDNA using the SV40 early promoter and polyadenylation signals and has been described previously (11).

**Tissue Culture**

CHO cells expressing the human PDGF receptor (B-type) cDNA using a pZipNeoSV(X) vector (26) were obtained from Drs. L. Clae-son-Welsh and C.-H. Heldin (University of Uppsala, Sweden). The cells were maintained in Ham's F12 medium supplemented with 10% fetal bovine serum and 0.25 mg/ml G418 (Geneticin, Gibco). The cells were transfected with plasmids using the calcium phosphate technique. After 3 days, the cells were passaged and selected using modified Eagle's minimum \(\alpha\) medium supplemented with 5% dialyzed fetal bovine serum, 0.1% G418. CHO cells that stably express wild-type or mutated EGF receptors were isolated using cloning rings and screened for the expression of EGF receptors by measuring the cell surface binding of \(^{125}\)I-EGF at 4°C.

**Analysis of \(^{125}\)I-EGF Binding**

CHO cells were seeded in 16-mm wells and grown to a density of 5 x 10\(^6\) cells/well. The cells were then incubated for 24 h in medium supplemented with 0.1% calf serum. The monolayers were then washed with 120 mM NaCl, 6 mM KCl, 1.2 mM CaCl\(_2\), 1 mM MgCl\(_2\), 25 mM HEPES (pH 7.4), 0.05 mM bovine serum albumin and incubated for 30 min at 37°C in the same medium. The cells were treated without or with phorbol ester or PDGF as 37°C and then rapidly cooled to 4°C. The binding of \(^{125}\)I-EGF to cell surface receptors was measured by incubation of the cells at 4°C for 2 h as described (7). Nonspecific binding was estimated in incubations containing a 500-fold excess of unlabeled ligand.

**Autophosphorylation of the EGF Receptor in Intact Cells**

CHO cells were grown in 35-mm dishes and washed with serum-free medium. The cells were then incubated at 37°C for 30 min in 1 ml of 120 mM NaCl, 6 mM KCl, 1.2 mM CaCl\(_2\), 1 mM MgCl\(_2\), 25 mM HEPES (pH 7.4), 0.05 mM bovine serum albumin and incubated without or with EGF or phorbol ester for defined times, and the EGF receptors were isolated from the cells by immunoprecipitation and polyacrylamide gel electrophoresis as described (7). The state of tyrosine phosphorylation of the EGF receptors was investigated by a Western blot procedure (27) using a monoclonal antiphosphotyrosine phosphate antibody (FY20) and a [\(^{125}\)I]-goat anti-mouse IgG antibody.

**Analysis of EGF Receptor Tryptic [\(^{32}\)P]-Phosphopeptides**

CHO cells were labeled with [\(^{32}\)P]phosphate for 18 h by incubation with phosphate-free Dulbecco's modified Eagle's medium supplemented with 0.1% calf serum and 200 \(\mu\)Ci/ml of [\(^{32}\)P]phosphate. EGF receptors were isolated from the cells by immunoprecipitation of detergent extracts with a polyclonal anti-EGF receptor antibody as described (7). The immunoprecipitates (100 µl) were reduced by heating at 60°C for 15 min in the presence of 80 µl of 10% NaDodSO\(_4\), 14 mM dithiothreitol. After cooling, the EGF receptors were alkylated by adding 40 µl of 0.4 M iodoacetamide, 0.25 M Tris-HCl (pH 8.8) and incubation at room temperature for 15 min. Subsequently, 80 µl of 15% glycerol, 20% 2-mercaptoethanol was added, and the sample was heated to 60°C for 15 min. After polyacrylamide gel electrophoresis, the gel slice containing the receptor was excised. The receptor was eluted with NaDodSO\(_4\) and precipitated with trichloroacetic acid as described (28). The sample was then digested with 1 µg of tosylphenylalanyl chloromethyl ketone-treated trypsin in 100 mM N-ethylmorpholine (pH 8.0). After 5 h, a second addition of trypsin was made, and the incubation was allowed to proceed for a further 19 h. Phosphopeptide mapping of the trypsin-digested EGF receptor was performed by reverse-phase HPLC using a Vydac C\(_8\) column (0.46 x 25 cm) equilibrated with 0.1% trifluoroacetic acid (7). Peptides were eluted by gradient elution with a flow rate of 1 ml/min. Nonspecific binding was estimated in incubations containing a 500-fold excess of unlabeled ligand.

**Phosphoamino Acid Analysis**

Phosphoamino acid analysis was performed by partial acid hydrolysis (1 h at 110°C in 6 M HCl) and thin layer electrophoresis by the method of Hunter and Selton (29) as described (5).

**Automated Amino-terminal Sequencing Analysis**

Sequence analysis of [\(^{32}\)P]phosphate-labeled peptides was performed in the presence of 4 nmol of myoglobin using a modified Beckman 490C liquid-phase sequenator and a 0.1 M Quadrol Program (Beckman 121078). Two precyclces were performed prior to the first cleavage. The anilinothiazolines were converted to phenylthiohydantoins by reaction in 25% trifluoroacetic acid at 56°C and were

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identified and quantitated by a modification of the reverse-phase HPLC procedure described by Zimmerman et al. (30) using acetonitrile. The radioactivity associated with the phenylthiohydantoin derivatives from the peptide that were released at each cycle was measured by liquid scintillation counting.

EGF Receptor Down-regulation

Two procedures were used to investigate the down-regulation of EGF receptors caused by incubation of cells with EGF.

Investigation of the Cell Surface Specific Binding of [125I]-EGF—CHO cells were seeded in 16-mm wells and grown to a density of 5 × 10^4 cells/well. The cells were washed with 120 mM NaCl, 6 mM KCl, 1.2 mM CaCl2, 1 mM MgCl2, 25 mM HEPES (pH 7.4), 30 mM bovine serum albumin, 10 mM glucose and incubated for 30 min at 37°C. The cells were treated without (control) or with 100 nM EGF at 37°C for 0.5, 1, 2, 3, and 4 h. The cell monolayers were then washed three times at 37°C over a period of 20 min and subsequently cooled to 4°C. The specific binding of [125I]-EGF to cell surface receptors was then measured at 4°C.

Investigation of the Expression of [35S]Methionine-labeled EGF Receptors—CHO cells were seeded in 35-mm wells and grown to a density of 2 × 10^5 cells/well. The cells were washed two times with methionine-free modified Eagle’s medium (Flow Laboratories) and incubated in 1 ml of medium supplemented with 1% fetal bovine serum and 10 μM [35S]methionine (30 μCi/nmol) for 18 h at 37°C. The cells were washed and subsequently incubated with 1 ml of Ham’s F12 medium in the absence (control) and presence of 100 nM EGF for 0.5, 1, 2, 3, and 4 h at 37°C. EGF receptors were isolated by immunoprecipitation, and the level of receptor expression was analyzed by polyacrylamide gel electrophoresis and fluorography as described (7).

Receptor-mediated Endocytosis of [125I]-EGF

CHO cells were seeded in 16-mm wells and grown to a density of 5 × 10^4 cells/well. Cells were washed with 120 mM NaCl, 6 mM KCl, 1.2 mM CaCl2, 1 mM MgCl2, 25 mM HEPES (pH 7.4), 30 mM bovine serum albumin and incubated for 30 min at 37°C in the same medium. The cells were incubated with 1 nM [125I]-EGF for 10, 20, 30, 40, 50, 60, and 90 min at 37°C and rapidly cooled to 4°C. The cells were washed without and with 50 mM NaCl, 150 mM glycine (pH 3.0) for 3 min at 4°C. Intracellular EGF was estimated by determination of cell-associated [125I]-EGF following acid washing (31). Cell surface [125I]-EGF was estimated by subtraction of the estimated intracellular radioactivity from the measured total cell associated [125I]-EGF. Non-specific binding and accumulation of [125I]-EGF was determined in experiments using a 500-fold excess of EGF.

Analysis of [3H]Thymidine Incorporation by CHO Cells

CHO cells were seeded in 16-mm wells and grown to a density of 5 × 10^4 cells/well. The cells were incubated for 18 h in medium supplemented with 0.1% calf serum. Growth factors were then added to the medium together with 5 μCi/ml of [3H]thymidine. After a further 24 h of incubation, the incorporation of radioactivity into acid-insoluble material was measured as described (32).

RESULTS

Characterization of EGF Receptor Phosphorylation Sites—Treatment of human fibroblasts with phorbol ester or with PDGF causes the increase in the phosphorylation state of the EGF receptor. Tryptic [32P]phosphopeptide mapping of the EGF receptor has demonstrated that the increase in phosphorylation occurs at multiple serine and threonine residues (5–7, 17–19). Analysis of the receptor tryptic [32P]phosphopeptides by reverse-phase high pressure liquid chromatography using a C18 column has demonstrated that there are several sites that are phosphorylated at a low stoichiometry (7). Most of the observed receptor phosphorylation is located on three tryptic [32P]phosphopeptides that elute from a C18 column at 7, 27, and 34% acetonitrile (5–7). The [32P]phosphopeptides eluting at 7 and 27% acetonitrile have been characterized previously and correspond to NH2-Glu-Leu-Val-Glu-Pro-Leu-Thr(P)-Pro-Ser-Gly-Glu-Ala-Pro-Asn-Glu-Ala-Leu-Leu-Arg-COOH, respectively (4, 5, 19, 33). The phosphorylated amino acids have been identified as Thr604 and Thr669 (4, 5, 19, 33). However, the tryptic [32P]phosphopeptide that we observe to be eluted at 34% acetonitrile (5–7) during reverse-phase chromatography has not been fully characterized.

In order to identify the [32P]phosphopeptide that elutes at 34% acetonitrile, experiments were designed to examine the properties of this peptide. (A) [32P]Phosphoamino acid analysis demonstrated the presence of [32P]phosphoserine (data not shown). (B) Incubation of the [32P]phosphopeptide in dilute acid caused quantitative cleavage of the peptide consistent with the presence of an Asp-Pro peptide bond (data not shown). (C) The [32P]phosphopeptide was a substrate for Staphylococcus aureus V8 protease and chymotrypsin (data not shown). (D) Automated Edman degradation of the [32P]phosphopeptide (27,000 cpm) caused the release of 35, 68, 476, 129, 81, 61, 69, 54, 62, 46, 51, 38, 37, and 26 cpm at cycles 1 through 15, respectively. These data indicate that the [32P]phosphopeptide contains a [32P]phosphoserine residue at position 3 from the amino terminus. Inspection of the cDNA sequence for the EGF receptor (34) indicated that there was only one predicted EGF receptor tryptic peptide that was consistent with the properties observed for the isolated [32P]phosphopeptide. The predicted phosphorylation site was Ser1047. To test the hypothesis that Ser1047 is an EGF receptor phosphorylation site, we substituted this residue with alanine using site-directed mutagenesis and expressed the mutated receptor in CHO cells. It was expected that the mutated [A1047]EGF receptor would be defective at the major site of serine phosphorylation. However, [32P]phosphopeptide mapping of the mutated [A1047]EGF receptor indicated that the major site of serine phosphorylation, located on a tryptic [32P]phosphopeptide that eluted from the reverse-phase column at 34% acetonitrile, was still present (data not shown). This result suggested that Ser1047 may not be a receptor phosphorylation site. To account for this unexpected result, we tested the hypothesis that the EGF receptor may be phosphorylated at Ser1046 in cells expressing the [A1047]EGF receptor. We therefore substituted Ser1046 with an alanine residue to examine the effect of this mutation on the phosphorylation state of the EGF receptor. It was observed that the phosphorylation of the [A1047]EGF receptor was similar to the wild-type EGF receptor (data not shown). However, [32P]phosphopeptide maps of the EGF receptor in which both Ser1046 and Ser1047 were substituted with alanine residues did not contain the tryptic [32P]phosphopeptide that elutes at 34% acetonitrile (Fig. 1). These data indicate that both Ser1046 and Ser1047 can serve as phosphorylation sites on the EGF receptor. Evidence that the EGF receptor can be phosphorylated at Ser1046 and Ser1047 in A431 cells has recently been reported by Heisemann and Gill (19).

Together, the data described above demonstrate that the major phosphorylation sites on the EGF receptor can be accounted for by Thr604, Thr669, Ser1046, and Ser1047. In order to examine the role of these phosphorylation sites for EGF receptor function, a series of mutant receptor cDNA constructs was prepared in which each of these sites was mutated by substitution with alanine. The wild-type and mutant EGF receptor cDNAs were then expressed in CHO cells together with the human B-type PDGF receptor (11).

Regulation of EGF Receptor Phosphorylation by Protein Kinase C—Experiments were performed to investigate the effect of protein kinase C activation on the phosphorylation state of the EGF receptor. CHO cells expressing wild-type and mutated EGF receptors were labeled with [32P]phosphate for 18 h and subsequently incubated without or with 10 nM...
FIG. 1. Characterization of the phosphorylation state of wild-type and mutated EGF receptors. Clones expressing wild-type and mutated EGF receptors were labeled with [32P]phosphate for 18 h. The cells were then treated without and with 10 nM PMA for 30 min at 37 °C. The EGF receptors were isolated by immunoprecipitation and polyacrylamide gel electrophoresis. Panel A presents a composite autoradiograph of dried polyacrylamide gels demonstrating the phosphorylation state of wild-type and mutated EGF receptors. Panels B–G present tryptic 32P-phosphopeptide maps of EGF receptors isolated from cells expressing wild-type EGF receptors (panel B), [A65']EGF receptors (panel C), [A65'A66']EGF receptors (panel D), [A64'Thr66']EGF receptors (panel E), [A65'Thr66']EGF receptors (panel F), and [A65'Thr66']EGF receptors (panel G). For clarity, 450 cpm has been added to each data point in maps obtained for EGF receptors isolated from PMA-treated cells. Each 32P-phosphopeptide fraction eluted from the reverse-phase column was investigated by phosphoamino acid analysis. The results of this analysis (S, phosphoserine; T, phosphothreonine; Y, phosphotyrosine) are presented in each panel.

PMA for 30 min. The EGF receptors were then isolated by immunoprecipitation and polyacrylamide gel electrophoresis. A marked increase in the phosphorylation state of the wild-type EGF receptor was observed after treatment of CHO cells with phorbol ester (Fig. 1A). An increase in EGF receptor phosphorylation was also observed after phorbol ester treatment of CHO cells expressing mutant receptors lacking either one or two phosphorylation sites. In contrast, phorbol ester caused only a small increase in phosphorylation of the EGF receptors when the four major phosphorylation sites (Thr654, Thr669, Ser1046, and Ser1047) were substituted with alanine residues (Fig. 1A).

To further characterize the sites of phosphorylation of the wild-type and mutant EGF receptors, the phosphorylation state of the receptors was investigated by 32P-phosphopeptide mapping. The EGF receptors were digested with trypsin, and the 32P-phosphopeptides obtained were analyzed by reverse-phase chromatography (Fig. 1). It was observed that the maps obtained for all EGF receptor mutants in which Thr654 was replaced by alanine lacked a 32P-phosphopeptide that eluted at 7% acetonitrile. Mutagenesis of Thr669 resulted in the loss of the 32P-phosphopeptide eluting at 27% acetonitrile. Furthermore, the 32P-phosphopeptide that elutes at 34% acetonitrile was not observed in maps of any of the EGF receptor
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mutants in which both Ser\textsuperscript{1046} and Ser\textsuperscript{1047} had been replaced with alanine residues (Fig. 1). No evidence for the phosphorylation of the mutant EGF receptors at sites that are not phosphorylated in the wild-type receptor was obtained (Fig. 1).

Examination of the \(^{32}P\)phosphopeptide maps of the mutant EGF receptor lacking the four major sites of phosphorylation (Thr\textsuperscript{654}, Thr\textsuperscript{662}, Ser\textsuperscript{1046}, and Ser\textsuperscript{1047}) indicated that this receptor contained several minor sites of phosphorylation on serine residues. Treatment of the CHO cells with phorbol ester caused a small increase in the labeling of all of these sites with \(^{32}P\)phosphate (Fig. 1). Two alternative hypotheses can account for the data obtained. First, it is possible that the 18-h incubation of the CHO cells with \[^{32}P\]phosphate was not sufficient to allow for the complete equilibration of all of the phosphate pools in the cell. Consequently, it is possible that the treatment of CHO cells with phorbol ester altered the specific radioactivity of the \(^{32}P\)phosphate labeling of the EGF receptor. A second hypothesis is that phorbol ester caused a small increase in the phosphorylation of several minor sites on the EGF receptor at a very low stoichiometry. As the increase in apparent phosphorylation observed was small and because the increase was found at all of the sites detected, further work will be required to distinguish between these hypotheses.

Regulation of \(^{125}\text{I}-\text{EGF}\) Binding to Cell Surface Receptors by PDGF and Phorbol Ester—The effect of mutagenesis of the major phosphorylation sites of the EGF receptor on the expression of the receptor was examined by investigation of \(^{125}\text{I}-\text{EGF}\) binding to cell surface receptors. Transfection of each of the cDNA constructs into CHO cells caused the expression of specific high affinity EGF binding sites at the cell surface. The binding isotherms were found to be curvilinear when plotted by the method of Scatchard (35). Analysis of the \(^{125}\text{I}-\text{EGF}\) binding isotherms using the computer program LIGAND (36) showed that a two-site model provided a significantly better description of the experimental data than a one-site model (p > 0.9). The results of this analysis for the wild-type EGF receptor and the \([\text{Ala}^{654}\text{A}^{662}\text{A}^{1046}\text{A}^{1047}]\)EGF receptor (Fig. 2) are summarized in Table I.

Treatment of cells with phorbol ester has been reported to cause an acute decrease in the high affinity binding of \(^{125}\text{I}-\text{EGF}\) to cell surface receptors. Transfection of each of the cDNA constructs into CHO cells caused the expression of specific high affinity EGF binding sites at the cell surface. The binding isotherms were found to be curvilinear when plotted by the method of Scatchard (35). Analysis of the \(^{125}\text{I}-\text{EGF}\) binding isotherms using the computer program LIGAND (36) showed that a two-site model provided a significantly better description of the experimental data than a one-site model (p > 0.9). The results of this analysis for the wild-type EGF receptor and the \([\text{Ala}^{654}\text{A}^{662}\text{A}^{1046}\text{A}^{1047}]\)EGF receptor (Fig. 2) are summarized in Table I.

The \(^{125}\text{I}-\text{EGF}\) binding isotherm (Fig. 2) was analyzed using the computer program LIGAND (36) to fit the data to a one-site and two-site model for the binding of \(^{125}\text{I}-\text{EGF}\) to cell surface receptors. The fit of the experimental data (mean ± S.E.) to the one-site model was significantly better (F test, p > 0.9).

| Site 1 | Site 2 |
|--------|--------|
| \(K_d\) | Sites/cell (x10\(^{-3}\)) | \(K_d\) | Sites/cell (x10\(^{-3}\)) |
| Wild-type [Thr\textsuperscript{654}, Thr\textsuperscript{662}, Ser\textsuperscript{1046}, Ser\textsuperscript{1047}] EGF receptor | 3.0 ± 1.6 | 176 ± 14 | 0.56 ± 0.20 | 12 ± 2 |
| PDGF | 4.3 ± 0.6 | 180 ± 30 | 4.7 ± 0.7 | 174 ± 24 |
| PMA | 7.0 ± 3.2 | 244 ± 38 | 0.15 ± 0.04 | 13 ± 3 |
| Mutated [Ala\textsuperscript{654}, Ala\textsuperscript{662}, Ala\textsuperscript{1046}, Ala\textsuperscript{1047}] EGF receptor | 9.9 ± 2.7 | 258 ± 35 | 0.17 ± 0.04 | 6.1 ± 2 |
| PDGF | 7.5 ± 2.8 | 231 ± 23 | 0.14 ± 0.05 | 3.0 ± 2 |

EGF receptors, a detailed study of PDGF and phorbol ester action was performed using CHO cells expressing the wild-type EGF receptor and the mutated \([\text{Ala}^{654}\text{A}^{662}\text{A}^{1046}\text{A}^{1047}]\)EGF receptor. In initial experiments, the effect of phorbol ester and PDGF on the \(^{125}\text{I}-\text{EGF}\) binding isotherm was investigated (Fig. 2). Analysis of the binding isotherms using the computer program LIGAND (36) indicated that phorbol ester and PDGF caused a decrease in the binding of \(^{125}\text{I}-\text{EGF}\) to high
affinity sites at the cell surface (Table I). The extent of the inhibition of 125I-EGF binding was observed to be greater for the wild-type receptor than for the mutated EGF receptor (Fig. 2 and Table I).

The time course of PDGF and phorbol ester action was investigated on the transmodulation of the wild-type and the mutated [A654A669A1045A1046A1047]EGF receptors. Similar results were obtained for both the wild-type and mutated EGF receptors (Fig. 4). Phorbol ester caused a rapid decrease in the binding of 125I-EGF to cell surface receptors which was sustained for at least 2 h (Fig. 4). In contrast to the sustained effect of phorbol ester, it was observed that the inhibition of 125I-EGF binding caused by PDGF was transient (Fig. 4). Maximal inhibition was observed after approximately 15 min of treatment with PDGF. EGF binding returned close to control levels after 1 and 2 h of treatment for the mutant and wild-type EGF receptors, respectively (Fig. 4).

The effect of PDGF and phorbol ester concentration was examined on EGF receptor transmodulation. Fig. 5 shows that the dose-response of PDGF and phorbol ester to inhibit 125I-EGF binding was similar for the wild-type and the [A654A669A1045A1046A1047]EGF receptors. At high concentrations of PDGF and phorbol ester it was observed that the maximum extent of inhibition of 125I-EGF binding was greater for the wild-type receptor than the mutated [A654A669A1045A1046A1047]EGF receptor (Fig. 5).

Regulation of EGF Receptor Endocytosis and Degradation—

The endocytosis of EGF receptors was investigated by incubation of cells expressing wild-type and mutated EGF receptors with 125I-EGF at 37 °C. The accumulation of 125I-EGF into an intracellular compartment after receptor-mediated endocytosis was examined by washing the cell monolayers at 4 °C with 50 mM NaCl, 150 mM glycine (pH 3.0) to remove 125I-EGF bound to the cell surface. Rapid endocytosis of 125I-EGF was observed in experiments using cells expressing wild-type and mutated receptors (data not shown). No significant differences were found between wild-type EGF receptors and receptors mutated at Thr654, Thr669, Ser1046, or Ser1047. Subsequent to endocytosis, the EGF receptor is degraded in lysosomes (2). The effect of EGF to cause down-regulation of the EGF receptor was investigated by two independent methods. First, the effect of EGF to cause a decrease in the expression of EGF binding sites at the cell surface was examined. Second, the effect of EGF on the expression of receptors by cells labeled with [35S]methionine was investigated. It was observed that EGF caused down-regulation of both wild-type and mutant receptors expressed in CHO cells (data not shown). We conclude that the substitution of Thr654, Thr669, Ser1046, and Ser1047 with alanine residues caused no significant alterations to EGF receptor down-regulation.

Regulation of EGF Receptor Tyrosine Protein Kinase Activity by Protein Kinase C—The tyrosine protein kinase activity associated with the intracellular domain of the EGF receptor is stimulated by the binding of EGF to the extracellular domain of the receptor. One of the substrates for the tyrosine protein kinase activity is the receptor itself. Consequently, the addition of EGF to cells causes an increase in the autophosphorylation of the receptor on tyrosine residues. EGF also causes an increase in the phosphorylation state of the EGF receptor at serine and threonine residues (37). To examine the role of the phosphorylation of serine and threonine residues on the function of the receptor, the effect of the substitution of Thr654, Thr669, Ser1046, and Ser1047 with alanine residues on EGF receptor autophosphorylation was investigated. Fig. 6 shows that EGF stimulated the autophosphorylation of the receptor on tyrosine residues in cells expressing wild-type and all of the mutated EGF receptors examined.

Treatment of cultured cells with phorbol ester has been shown to inhibit the tyrosine protein kinase activity of the EGF receptor (12, 13). To evaluate the role of the phosphoryl-
Role of EGF Receptor Phosphorylation during Signal Transduction—The role of EGF receptor phosphorylation in the signalling pathway for cellular proliferation was investigated by measurement of the growth factor-stimulated incorporation of [\( ^{3}H \)]thymidine into DNA. Fig. 7 shows that the wild-type EGF receptor and the mutated [\( A^{654}A^{666}A^{1046}A^{1047} \)]EGF receptor were capable of transducing a mitogenic signal. EGF caused only a small increase in the incorporation of [\( ^{3}H \)]thymidine by cells expressing the wild-type EGF receptor. In contrast, a marked increase in the incorporation of [\( ^{3}H \)]thymidine was observed after EGF treatment of CHO cells expressing the mutated [\( A^{654}A^{666}A^{1046}A^{1047} \)]EGF receptor. Investigation of the role of individual phosphorylation sites indicated that the marked increase in EGF receptor signalling could be accounted for by the substitution of Thr\(^{654} \) with an alanine residue (data not shown, Refs. 10 and 11). Livneh et al. (10) have proposed the hypothesis that the increased mitogenic responses mediated by the EGF receptor lacking Thr\(^{654} \) is caused by a defect in the desensitization of the EGF receptor. While receptor phosphorylation may be related to the functional desensitization of signalling (38), the data presented here demonstrate that mitogenic signal transduction by the EGF receptor does not require Thr\(^{654} \), Thr\(^{666} \), Ser\(^{1046} \), or Ser\(^{1047} \) (Fig. 7).

In further experiments, the effect of PDGF to stimulate the growth of CHO cells was examined. It was observed that PDGF increased the EGF-stimulated incorporation of [\( ^{3}H \)]thymidine by cells expressing both wild-type and mutated EGF receptors (Fig. 7). Thus, the mechanism of PDGF action to stimulate cellular proliferation does not require the phos-

![Diagram](image_url)
there are three major tryptic phosphopeptides that elute from phosphorylation of the EGF receptor is mechanistically re-

associated with protein kinase activity of the EGF receptor. Associated with the phosphorylation of Thr669 (19, 33), and Ser1047 (Fig. 1 and Ref. 19), respectively. be a substrate for a calmodulin-dependent protein kinase (19). phosphorylation of the EGF receptor at Thr654, Thr669, Ser1046, and Ser1047. DISCUSSION

Treatment of cultured cells with phorbol ester or with PDGF causes a rapid decrease in the high affinity binding of EGF to cell surface receptors and an inhibition of the tyrosine protein kinase activity of the EGF receptor. Associated with these changes in EGF receptor function is an increase in the extent of serine and threonine phosphorylation of the EGF receptor at several sites. The role of receptor phosphorylation is incompletely understood, but it has been proposed that the phosphorylation of the EGF receptor is mechanistically related to the process of EGF receptor transmodulation (reviewed by Schlessinger, Ref. 2). The purpose of the study reported here was to test this hypothesis. The approach that we have taken was to examine the effect of the mutagenesis of the major sites of phosphorylation on the EGF receptor. Phosphopeptide mapping of the wild-type EGF receptor isolated from phorbol ester-treated cells demonstrates that there are three major tryptic phosphopeptides that elute from a reverse-phase C18 column at 7, 27, and 54% acetonitrile (Fig. 1). These phosphopeptides have been characterized, and the phosphorylated residues have been identified as Thr654 (4, 5), Thr669 (19, 33), and Ser1047 (Fig. 1 and Ref. 19), respectively. The mechanisms by which these sites are phosphorylated in phorbol ester-treated cells are distinct. Thr654 is a substrate for protein kinase C (4, 5), Thr669 is a substrate for a novel growth factor-stimulated protein kinase (33), and Ser1047 may be a substrate for a calmodulin-dependent protein kinase (19). The role of the phosphorylation of the EGF receptor at these major sites of phosphorylation was examined by replacement of these residues with alanine.

Phosphopeptide mapping of EGF receptors lacking Thr654 resulted in the loss of the phosphopeptide that eluted from the reverse-phase column at 7% acetonitrile, as expected (Fig. 1). Similarly, the mutagenesis of Thr669 caused the loss of the peptide eluting at 27% acetonitrile. However, this result was not expected because Heisermann and Gill (19) have reported that Ser1047, which is located adjacent to Thr669 in the phosphopeptide, is also a substrate for phosphorylation. The replacement of Thr669 with an alanine residue therefore appears to prevent the phosphorylation of Ser1047. As Ser1047 was not phosphorylated on the [A669]EGF receptor (Fig. 1), the effect of site-directed mutagenesis of Ser1047 was not investigated in the present study. In contrast to the results obtained for the mutagenesis of Thr654 and Thr669 (Fig. 1), the substitution of Ser1047 with an alanine residue caused no significant alteration to the phosphopeptide map of the EGF receptor. This result could be accounted for by the phosphorylation of Ser1047 in the [A669]EGF receptor (19). To investigate this possibility, Ser1047 was replaced with an alanine residue. It was observed that the phosphopeptide map of the [A669]EGF receptor was similar to that of the wild-type EGF receptor. However, phosphopeptide maps of the [A669A1047]EGF receptor lacked the phosphopeptide that elutes at 34% acetonitrile from the HPLC column (Fig. 1). These data indicate that both Ser654 and Ser1047 are sites of EGF receptor phosphorylation (19). Substitution of all of the major sites of phosphorylation (Thr654, Thr669, Ser1046, and Ser1047) with alanine residues caused a marked decrease in the level of phosphorylation of the EGF receptor (Fig. 1). Phosphopeptide mapping demonstrated that the [A669A1047]EGF receptor lacked all of the major sites of phosphorylation, but retained phosphorylation at several minor sites that were also observed in maps of the wild-type EGF receptor.

Inhibition of High Affinity EGF Binding by Protein Kinase C—EGF receptor transmodulation was examined by investigation of the high affinity binding of lz51-EGF to cell surface receptors. A decrease in the binding of lz51-EGF was observed when cells were incubated with phorbol ester or PDGF. Similar results were observed for cells expressing wild-type receptors and mutated EGF receptors (Fig. 3). Thus, phorbol ester and PDGF inhibit lz51-EGF binding to cells expressing mutated EGF receptors that lack the major sites of EGF receptor phosphorylation (Thr654, Thr669, Ser1046, and Ser1047). We conclude that the mechanism of action of PDGF and phorbol ester to cause a decrease in the apparent affinity of the EGF receptor is independent of receptor phosphorylation at Thr654, Thr669, Ser1046, and Ser1047.

Further studies are required to identify the mechanism of EGF receptor transmodulation. Two general hypotheses can be presented to account for the mechanism of EGF receptor transmodulation. 1) Transmodulation of the EGF receptor may be caused by phosphorylation of the receptor. The data reported here demonstrate that the major sites of EGF receptor phosphorylation are not mechanistically related to transmodulation. Therefore, if receptor phosphorylation is relevant to transmodulation, the stoichiometry of phosphorylation at the potential regulatory sites is extremely low (Fig. 1). It is also possible that transmodulation is caused by the phosphorylation of a protein that interacts with the receptor. 2) Phosphorylation may not account for EGF receptor transmodulation. The decrease in the apparent affinity of the EGF receptor may therefore be accounted for by the interaction of the receptor with other cellular components. Rigorous tests of these hypotheses are required for future progress toward the present study. In contrast to the results obtained for the mutagenesis of Thr654 and Thr669 (Fig. 1), the substitution of Ser1047 with an alanine residue caused no significant change in the phosphorylation of Thr669. However, replacement of Thr669 with alanine abolished the minor phosphorylation of Ser1047 observed. The phosphorylation of Ser1047 in vitro therefore requires Thr669 (Northwood, I. C., and Davis, R. J., manuscript submitted for publication). These in vitro data are consistent with the results of phosphopeptide mapping of the [A669]EGF receptor phosphorylated in vivo (Fig. 1).
the understanding of the mechanism of EGF receptor trans-
mobilization.

Inhibition of EGF Receptor Tyrosine Protein Kinase Activity by Protein Kinase C—Treatment of cells with phorbol ester causes an inhibition of the EGF receptor tyrosine protein kinase activity (12, 13). The role of EGF receptor phosphorylation during this process was examined by investigation of the effects of the substitution of the major receptor phosphorylation sites (Thr654, Thr661, Ser1046, Ser1047) with alanine residues. It was observed that phorbol ester caused an inhibition of the autophosphorylation of EGF receptors lacking Thr654 (Fig. 6). Similar results were observed for EGF receptors lacking Ser1046 and Ser1047 (Fig. 6). These data demonstrate that the phosphorylation of the EGF receptor at Thr654, Ser1046, and Ser1047 is not required for the inhibition of the EGF receptor tyrosine protein kinase activity caused by phorbol ester. In contrast, no significant inhibition of receptor autophosphorylation was observed after substitution of Thr654 with alanine (Fig. 6). This result indicates that Thr654 is required for the inhibition of EGF receptor autophosphorylation.

The possible functional interaction between the phosphorylation of Thr654 and the phosphorylation of the EGF receptor at other sites was examined. The approach that we used was to compare the properties of receptors with single point mutations with receptors in which two or more phosphorylation sites were replaced with alanine residues. It was observed that phorbol ester did not inhibit the autophosphorylation of any of the mutated receptors that lack Thr654 (Fig. 6). In contrast, phorbol ester caused an inhibition of the autophosphorylation of the wild-type EGF receptor and all of the mutated receptors with Thr654 (Fig. 6). These data indicate a primary role for Thr654 compared with other receptor phosphorylation sites during regulation of the receptor tyrosine protein kinase activity by phorbol ester.

Evidence to support the hypothesis that phosphorylation of Thr654 by protein kinase C accounts for the regulation of the receptor tyrosine protein kinase has been obtained from the investigation of the effects of PDGF on human fibroblasts. Comparison of different cell lines has demonstrated that there is a correlation between the ability of PDGF to cause phosphorylation of Thr654 and to cause an inhibition of the EGF receptor tyrosine protein kinase activity. Transmobilization of the EGF receptor caused by treatment of WI-38 human fetal lung fibroblasts with PDGF causes both an inhibition of the EGF receptor tyrosine protein kinase activity and an increase in the state of phosphorylation of Thr654 (6, 7). In contrast, treatment of FS4 human skin fibroblasts with PDGF does not increase Thr654 phosphorylation, and EGF receptor transmobilization occurs without an inhibition of the receptor tyrosine protein kinase activity (39). Thus, the ability of PDGF to cause an inhibition of the EGF receptor tyrosine protein kinase activity correlates with phosphorylation of the EGF receptor at Thr654 (6, 7, 39). More direct evidence that supports the hypothesis that Thr654 phosphorylation modulates the EGF receptor tyrosine protein kinase activity has been obtained from the results of in vitro reconstitution experiments in which the phosphorylation of Thr654 by protein kinase C is associated with an inhibition of the receptor tyrosine protein kinase activity (13–15). Together, these data strongly support the hypothesis that phosphorylation of Thr654 represents an important mechanism of regulation of the EGF receptor tyrosine protein kinase activity. However, it has recently been reported that the EGF receptor tyrosine protein kinase activity can be regulated by additional mechanisms. Treatment of cells with the non-phorbol ester tumor promoter thapsigargin causes a decrease in the tyrosine protein kinase activity of the EGF receptor without causing phosphorylation of the receptor at Thr654 (16). The regulation of the EGF receptor tyrosine protein kinase activity caused by thapsigargin therefore occurs by a mechanism that is independent of Thr654 phosphorylation. Further studies are warranted to identify regulatory mechanisms that are independent of Thr654 and to examine their physiological significance.

Role of EGF Receptor Phosphorylation during Mitogenic Signal Transduction—The role of EGF receptor phosphorylation was examined by comparison of the growth factor-stimulated incorporation of [3H]thymidine into DNA by cells expressing wild-type EGF receptors with cells expressing mutated EGF receptors that lack all of the major sites of serine and threonine phosphorylation (Thr654, Thr661, Ser1046, and Ser1047). It was observed that EGF caused an increase in the incorporation of [3H]thymidine by cells expressing wild-type and mutated EGF receptors (Fig. 7). However, the mitogenic effects of EGF mediated by the mutated receptor lacking phosphorylation sites was observed to be significantly greater than that mediated by the wild-type receptor. The modest effect of EGF on stimulating [3H]thymidine incorporation by cells expressing wild-type EGF receptors is consistent with previous reports of functional desensitization of EGF receptor signalling (38, 40). Investigation of the role of individual phosphorylation sites indicated that the marked increase in EGF receptor signalling could be accounted for by the mutagenesis of Thr654. Livneh et al. (10) have reported that the increased mitogenic responses mediated by the EGF receptor lacking Thr654 is caused by a defect in the desensitization of the EGF receptor. The data reported here are consistent with this hypothesis.

Conclusions—A summary of the conclusions about the role of EGF receptor phosphorylation that we have drawn from the results of this study is presented in Fig. 8. Phosphorylation of...
Role of EGF Receptor Phosphorylation

of the EGF receptor at Thr<sup>654</sup> plays a critical role in the regulation of the tyrosine protein kinase activity of the EGF receptor. In contrast, the mechanism of regulation of the apparent affinity of the EGF receptor caused by PDGF and phorbol ester is independent of the phosphorylation of the EGF receptor at all of the major sites of serine and threonine phosphorylation.

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